Behavioural Effects of Prenatal Exposure to Aspartame in Rats

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Table of Contents

List of Tables

List of Figures

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Abstract

Aspartame (Nutrasweet ®, APM) is a widely used artificial sweetener synthesised from aspartate and phenylalanine, both of which are freely available in the normal diet but known to exert toxic and teratogenic effects when consumed in large quantities. While APM's protagonists claim that it is the most thoroughly researched food additive ever, there have been only a handfull of studies on it's behavioural effects. This study examined the behavioural teratogenic effects of APM in the offspring of rats which were exposed throughout pregnancy to solutions containing 0.05% or 0.10% APM or a buffered control solution. The animals were tested in the Hughes exploration box and the 12-arm radial maze using six arms baited at the beginning of each trial and lowering guillotine doors each time the rat returned to the centre of the maze to prevent the rats from adopting response strategies. In the Hughes exploration box, control and APM animals showed a preference for novelty. There were no difference between groups on measures of exploration and ambulation. In learning the radial arm maze task, there were no differences in the number of errors made by the groups and all animals learned the task to a high degree of proficiency. However, during the first half of the 40 sessions and relative to controls, the rats prenatally exposed to the 0.10% APM solution took significantly less mean time to enter the arms of the maze for the first six arm entries; the time taken by the 0.05% APM group generally fell between that of the control and 0.10% APM group. These results suggest that APM may have a dose-dependent effect on the time taken by rats to enter an arm in the radial arm maze. The validity of these results is limited by some methodological considerations, such as the relatively low genetic diversity of the groups, and this study is in need of replication. Despite these caveats, this study is significant as it is the only behavioural teratology study with APM to use an adequate cross-fostering procedure, a low dose of APM and report a dose-dependent behavioural effect. The discussion offers suggestions for future research that could improve our knowledge of the possible behavioural teratogenicity of APM.

Chapter 1

Introduction

The present study examined the behavioural toxicology and behavioural teratology of aspartame (Nutrasweet®), the latest substance to be widely used as a sweet alternative to the large quantities of sugar consumed by in the western diet. Aspartame has about the same calorific value as sugar (about 4 Kcal/g) and a perceived sweetness of between 160 and 200 times the sweetness of sugar. This intense sweetness means that only small quantities, offering few calories, are needed to sweeten food. While sugar may supply as much as 17 percent (Pardridge, 1986) to 25 percent (Roak-Foltz and Leveille, 1984) of our total daily calories, it is held responsible for causing obesity, dental caries (Bowen, 1984) and exacerbating diabetic conditions (Porikos and Van Itallie, 1984). Aspartame is not the first artificial sweetener to be widely used, but it is hoped that it is safer than previous sugar substitutes such as saccharin and cyclamate. Saccharin has been used as a sweetener since 1907 but in 1977 it was almost withdrawn from the American market after it was shown to cause cancer in laboratory animals (Gossel, 1984). Consumer protests lead to an American Congressional act which prevented the American Food and Drug Administration from banning saccharin. Saccharin is still used widely in foods, despite it's metallic aftertaste. Cyclamate was a popular sweetener in the 1960s but it was withdrawn from the American market in 1970 after it was shown to be carcinogenic (Gossel, 1984).

Aspartame (L-aspartyl-L-phenylalanine methyl ester; APM) is a synthetic di-peptide composed of the amino acids aspartic acid (aspartate, ASP) and phenylalanine (PHE) in a methyl ester. When ingested, APM is hydrolysed in the intestine to aspartylphenylalanine and methanol. The aspartylphenylalanine peptide is hydrolysed to ASP and PHE, mostly in the intestine but also in the

intestinal mucosa and in red blood cells (Tobey and Heizer, 1986; Opperman, 1984). Aspartame has been found to be metabolized in the same way in all mammalian species studied (Ranney, Opperman, Muldoon and McMahon, 1976). The catabolism of APM produces 40 percent ASP, 50 percent PHE and 10 percent methanol.

All three APM catabolites are found in large quantities in any normal healthy diet. Large amounts of ASP and PHE are found in dietary proteins, including those in human milk (Baker, 1984). Aspartate is also available in some foods as a free amino acid. There is more methanol in fresh fruit juice than in an equal volume of APM sweetened beverage. Aspartate is similar to glutamate (glutamic acid, GLU), which is also found in a range of foods. Both ASP and GLU are known chemically as dicarboxylic amino acids and both are involved in a range of essential metabolic processes in the body, including the Krebs (energy metabolism) cycle and the urea (nitrogen) cycle (Stegink, 1984a). Glutamate is widely used as a flavour enhancing food additive in the form of monosodium glutamate. Throughout this study, ASP and GLU will be considered together.

APM is currently used in New Zealand as a 'low calorie' sweetener in 'diet' soft-drinks, as a free flowing and tablet sweetener (Equal®) and in 'diet' chewing gum. In 1984, the New Zealand Department of Health approved APM for use as a food additive. This decision was based on the approval of APM for use in foods by the United States Food and Drug Administration (FDA) in 1981 (Hayes, 1981) and 1983 (FDA, 1983)[1]. In America, APM is permitted as a food additive in a wide range of dry foods and beverages (Maher, 1986). Since APM was introduced in America, consumption has risen exponentially to 8000 tons in 1986 (Wurtman, 1987) with over 20

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[1] Personal communication from Dr. J. Fraser, Chief Scientist, Department of Health, Wellington, New Zealand; and see Parker (1988).

billion cans of APM sweetened soft drink being consumed by over 100 million Americans each year (Metzenbaum, 1987).

1.1. American Approval of Aspartame

The FDA took over eight years to assess the safety of using APM as an intentional food additive. The FDA considered over 100 studies relating to APM safety, including clinical studies on humans and toxicological testing on animals, and the FDA produced a series of controversial regulatory decisions (Hattan, Henry and Montgomery, 1983).

Aspartame was initially approved in America in 1974. Soon after, the FDA requested the voluntary postponement of marketing and then withdrew the authorization. Amongst allegations of fraud and while considering legal action (see Olney, 1987), the validity of some of the research data submitted by the petitioner (G.D. Searle and Co.) was subjected to independent validation. Formal objections to the approval led to the establishment of a Public Board of Inquiry to examine APM's safety. The Board reported that the evidence for the safety of APM was inconclusive and that it should not be approved for marketing. However the American Bureau of Foods and the newly appointed Commissioner of the FDA, Arthur Hayes, did not agree. They concluded with "...reasonable certainty..." that APM was safe and it was approved for market release (Hayes, 1981). Hayes commented that he was "...not prepared to say that there was no risk ... but ... it had been demonstrated that there was not a significant risk..." (Smith, 1981).

1.2. Reported Problems with Aspartame

APM is composed of natural food agents and has been claimed to be the most thoroughly studied of all food additives (e.g., Gossel, 1984; Stegink and Filer, 1984). However, by the end of 1987 over 4000 consumer complaints had been made to the FDA relating to APM use (Metzenbaum, 1987). In 1984 the American Centre for Disease Control (see Maher,1986; Pardridge, 1986) published an investigation of 517 consumer complaints relating to APM use. The complaints were primarily related to either the nervous system (mood changes, insomnia, seizures, menstrual irregularities) or the gastro-intestinal system (abdominal pain, nausea, diarrhea). APM ingestion has also been associated with dermatological complaints (Kulczycki, 1986; Novick, 1985) and panic attacks (Drake, 1986).

Roberts (1987)[2] studied 551 people who had had "bizarre" and "bothersome" reactions to APM. Almost half complained of severe headaches, about one third suffered from severe dizziness and eleven lost sight in one or both eyes. Others had seizures and memory problems. His report to the 154th annual meeting of the American Association for the Advancement of Science suggested that APM posed "an imminent health hazard" and it should be investigated as soon as possible. Other recent calls for the re-assessment of APM's safety have come from the American Community Nutrition Institute (see Parker, 1988) and several expert witnesses before the 1987 United States Congressional Senate Committee on Labour and Human

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[2] Roberts (1987) is a statement to the 1987 American Congressional Hearing on Nutrasweet based on a study published in two American Health Sector magazines. His report to the American Association for the Advancement of Science was reported anonymously in "New Scientist" magazine, volume 117, page 33, 1988).

Resources hearing on APM health and safety concerns (e.g., Olney, 1987; Elsas, 1987; Wurtman, 1987).

1.3. Potential Problems with Aspartame

Most of the early debate over the safety of APM resulted from a study submitted by the petitioner (G.D. Searle) which suggested that APM may cause brain cancer in rats. While this issue has not been resolved to the satisfaction of some (e.g. Olney, 1987), it is beyond the scope of the present study and will not be considered further.

The other main issue considered during the regulatory process was whether "...the ingestion of aspartame, alone or together with glutamate, poses a risk of *contributing* to mental retardation, brain damage , or undesirable effects on the neuroendocrine system..." (Hattan *et al.*, 1983, p. 42). This concern was related to the known adverse effects of ASP and PHE ingestion under certain conditions and it is these issues that are the main focus of the present study. Although methanol, the other product of APM metabolism, is known to be toxic, it is widely assumed that there is no reason to suspect that the low quantities of methanol released by APM metabolism will be toxic (see Pardridge, 1986).

The remainder of this section examines the risks associated with the ingestion of APM within the context of the known adverse effects of it's two principal components and metabolites, namely PHE and ASP. Also, a short section will examine the potential risks associated with the intact APM peptide.

1.3.1. Estimated Daily Intake of Aspartame

As any substance can be harmful if an organism is exposed to large enough quantities of it, the safety of APM depends on the amount that is likely to be consumed as well as the amount that is likely to be harmful. The FDA determines the maximum acceptable intake of a food additive by dividing the minimum dose required to produce adverse effects in experimental animals by a safety factor. This safety factor is usually 100, which is an arbitrary number thought to represent a realistic safety margin (Hattan *et al.*, 1983). The FDA decided that the acceptable daily intake of APM is 50 mg/Kg/day. However, what is often neglected when comparing dose levels across species is that there is an inverse relationship between metabolic rate and body mass in mammals (Pardridge, 1986). Rats metabolize PHE six times faster than do humans, so failure to consider this difference would divide the safety factor to less than 17. It is unclear whether this issue was taken into account in the FDA's decision.

If the expected daily intake of the substance is less than the acceptable daily intake, then the substance is considered to be safe. The American regulatory authorities calculated the expected daily intake of APM from market research data reporting the food consumed in 4000 households. They assumed that sugar was completely replaced by APM, where possible, and calculated a mean consumption of 11.1 mg/Kg/day APM with a 99th percentile level of 34 mg/Kg/day. From these figures APM was considered safe, but it remains controversial whether these consumption figures are realistic. Pardridge (1987) notes that 50 mg/Kg/day of APM can be ingested by consuming five average servings of APM per 50 pounds of body weight per day. Children, with their low body weights and sweet tastes, can easily consume this amount. A 20 Kg child who consumes just one quart of APM sweetened soft drink will receive an acute dose of 25 mg/Kg (Pardridge 1986). Frey (1976) found that seven to twelve year old children consumed up to 77 mg/Kg/day when given free access to APM containing products. Even adults have been reported to consume up to 36 mg/Kg/day of APM with a mean daily intake of 20 mg/Kg/day (Porikos and Van

Itallie, 1984). These figures suggest that the FDA estimation of daily intake of APM is unrealistictly low and that the actual intake figure may well be in excess of the 50 mg/Kg/day figure which the FDA considers to be a safe level of intake.

1.3.2. Phenylalanine

Most of the concern about the possible harmful effects of APM has focussed on PHE. Phenylalanine is an essential amino acid that mammals can not synthesize and have to obtain from food. Phenylalanine is degraded through a variety a pathways, though mostly it is hydrolysed to tyrosine. Tyrosine is also an important amino acid and a precursor in catecholamine and melanin synthesis (Harper, 1984). Dietary PHE is a major source of tyrosine. The ingestion of even low doses of APM causes a sharp and significant increase in PHE plasma levels (Harper, 1984). Phenylalanine is transported readily through the blood-brain barrier (Pardridge, 1986) and the developed placenta concentrates PHE at a ratio of about 2:1 on the side of the fetus (Stegink, Pitkin and Reynolds, 1979).

Research into the human gene linked disorder of phenylketonuria (PKU) has shown that high plasma PHE levels have a detrimental effect on the developing brain. Phenylketonuria affects about one person in 10000 - 15000 and involves a deficiency in the PHE hydrolysing enzymes in the liver which causes high plasma PHE levels. If these high PHE levels are not treated through dietary modifications, the young child with PKU will develop severe mental retardation during the first few years of life. However, the child of a woman who has non-PKU induced high PHE levels during pregnancy may also be born with brain damage, microcephaly, low birth weight, and congenital heart disease. Levy and Waisbren (1983) studied mothers with PKU or non-PKU hyperphenylalaninemia and their children. When controlling for maternal IQ differences, they found a high correlation between childrens' IQ and maternal PHE levels (r= -0.82) in those children who had neither PKU nor non-PKU

hyperphenylalaninemia. Thus, a five fold increase in maternal PHE levels was associated with a 10 point reduction in the childs' IQ. Maternal plasma PHE levels above 60 µmol/dl PHE were likely to have noticeable adverse effects on the child and PHE levels greater than 110 µmol/dl were very likely to cause mental retardation. This study shows that an increase in maternal PHE levels can be harmful to the fetus regardless of the cause of the PHE increase. From their findings, Levy and Waisbren suggested that maternal PHE levels may be dangerous to the fetus only once a threshold level is exceeded, but Pardridge (1986) maintains that there is most probably a linear relationship between maternal plasma PHE levels and effects on the fetus. The effects produced by less than 60 µmol/dl PHE are likely to be subtle and less obvious than the effects of higher maternal PHE levels and these subtle effects may easily go un-noticed.

Animal models of PKU have been developed using behavioural measures of learning as measures of cognitive impairment (see Vorhees, Butcher and Berry, 1981). Early animal models of PKU showed that administration of PHE could impair discrimination learning (Schalock and Klopfer, 1967;), avoidance learning and operant learning (Chamove and Harlow, 1973; Hess, Paulsen, Muller and Carlton, 1966) in rats and monkeys. Current animal models of PKU, which co-administer PHE and an inhibitor of PHE and tyrosine metabolism, report similar impairments (Vorhees *et al.*, 1981).

Elevated PHE levels have been found to cause changes even in human adults. A three-fold increase in the normal PHE levels of 3 to 5 µmol/dl has been shown to result in quantitative changes in EEG patterns and a six-fold increase has been shown to induce a 10 percent change in performance on a choice time experiment (Elsas, 1987).

Risks from Aspartame Released Phenylalanine

Phenylalanine is an important part of any normal healthy diet. One litre of APM sweetened soft-drink contains about 400 mg of PHE which is about the same amount of PHE in either one egg, one ounce of cheddar cheese or six ounces of baked beans (Yellowlees, 1983). The ingestion of the FDA's acceptable daily intake of 50 mg/Kg APM can double the normal PHE plasma levels (Matalon, 1987) but the ability of PHE to reach the brain depends on the amount of competition from other large neutral amino acids for transport through the blood-brain barrier in a common carrier system. The large neutral amino acids found in dietary proteins can compete with normal dietary PHE for blood-brain barrier transport to the extent that brain PHE levels may decrease follow protein intake. But the PHE released from APM is readily able to enter the brain as often the APM released PHE is not accompanied by competing large neutral amino acids. Thus, brain changes in PHE from APM ingestion can be in the opposite direction from the changes which result from the ingestion of the above mentioned foods (Wurtman and Maher, 1984; Wurtman, 1983). Rather than being biologically innocuous, Maher (1986) has claimed that APM is the food most "capable of selectively enhancing brain PHE levels ... ever consumed".

The competition between neutral amino acids for blood-brain barrier transport means that PHE can exert an indirect influence on the neurochemistry of the brain through changing the availability of other neutral amino acids which are neurotransmitter precursors (Pardridge, 1986; Fernstrom and Wurtman, 1972). Wurtman (1983) notes that when APM is consumed with carbohydrates and without protein, the rise in brain PHE levels can be double that when the carbohydrate is not consumed. Also, the normal changes in brain tryptophan levels that follow carbohydrate ingestion can be disrupted. Tyrosine and tryptophan are precursors in catecholamine and serotonin metabolism respectively. Experiments with rats have

found that abuse doses of APM and doses as low as 200 mg/Kg APM can increase brain PHE, tyrosine and catecholamine levels and block the normal increase in brain tryptophan and serotonin which follows carbohydrate ingestion (Yokogoshi and Wurtman, 1984; Wurtman, 1983; Coulombe and Sharma, 1986; Fernstrom, Fernstrom and Gillies, 1983). Autopsies of patients with PKU have found reduced amounts of tyrosine and tryptophan as well as "substantial" reductions in serotonin, dopamine and noradrenaline (McKean, 1972). However, the FDA (1987) considered that these studies into neurochemical changes following APM ingestion are inadequate as they did not take into account the natural variation in brain levels of monoamines and their precursors and they did not relate neurochemical changes to neurobehavioural effects. It is noteworthy that the FDA recently agreed to fund research to investigate this issue.

Groups Vulnerable to the Effects of Aspartame Released Phenylalanine

It is well recognized that PHE poses a health risk to certain individuals, such as those who have PKU. To enable these individuals to avoid consuming PHE in APM, the labels on products which contain APM are required to warn people who have phenylketonuria that the product contains PHE. While this may be adequate warning for PKU sufferers, who are usually aware of their condition, individuals with a heterozygous PKU gene are usually unaware of the fact that they have about half the normal PHE hydrolysing capacity. Consequently, the doses of APM that are required to cause two and four fold increases in plasma PHE levels in individuals with normal PHE hydrolysing capacity will, respectively, cause three and ten fold increases in plasma PHE levels in individuals with a heterozygous PKU gene (Matalon, 1987). Furthermore, acute plasma PHE elevations remain higher for longer in individuals with a heyerozygous PKU gene than in individuals with normal PHE hydrolysing capacity. As between two and ten percent of the population may have a heterozygous

PKU gene (Pardridge, 1986), a significant proportion of the population may be endangering their health by consuming APM without realising the risk.

Even individuals with normal PHE levels and PHE hydrolysing capacity may be at risk from APM released PHE. Phenylalanine plasma concentrations need to be only three times normal levels to induce cognitive changes (page 7) and PHE plasma levels are doubled by only 50 mg/Kg/day APM - a level of consumption which is easily exceeded (see Section 1.3.1). Also, as the acute ingestion of only 34 mg/Kg APM can cause a five fold increase in plasma PHE levels in fasting individuals (Elsas, 1987), a more pressing problem is that people who intentionally restrict their food intake may be particularly vulnerable to the effects of the APM released PHE. This is precisely the group of people who are attracted towards the use of a 'low calorie' sweetener.

It is well recognized that the developing fetus is especially sensitive to the adverse effects of environmental and metabolic toxins (Vorhees and Butcher, 1982), including high maternal plasma levels of PHE (see page 7). The fetus may also be vulnerable to the effects of APM released PHE. Elsas (1987) claims that the combined effects of the placental barrier and the developing fetal blood-brain barrier may concentrate PHE in the fetus to as much as four to six times the concentration present in the maternal plasma. Hence a maternal plasma concentration of 15 μmol/dl PHE could lead to 90 μmol/dl in the fetal brain, which is a high enough concentration of PHE to kill neural cells, at least in tissue cultures (Elsas, 1987). Also, the fetus metabolizes PHE less effectively than the adult so elevated PHE levels will remain in the fetus after the maternal PHE levels have returned to normal limits. As noted above (this Section), plasma PHE concentrations can reach 15 μmol/dl after consuming only 50 mg/Kg of APM and moderate elevations in maternal PHE levels may produce subtle dose dependent effects in their children. The western preoccupation with a slim female body shape may pose an extra hazard to the fetus by encouraging pregnant women to consume extra amounts of APM containing foods during pregnancy in an effort

to restrict their weight gain (Pardridge, 1986). As an insult to the developing fetus will have an effect which usually lasts for the lifetime of the animal, the group most affected by APM released PHE may be the unborn.

1.3.3. Aspartate

While most researchers who are concerned with the safety of APM have focussed their attention on the PHE moiety, Olney (see Olney, 1984, 1987; Olney, Labruyere and deGubareff, 1980) has consistently maintained that APM ingestion may be harmful through the action of it's ASP moiety. Claims that APM-released ASP may pose a health risk are more controversial than similar claims about PHE and therefore the following section will consider the relevant issues in some detail.

Aspartate (ASP) and the structurally and functionally similar glutamate (GLU) are found in large quantities in any healthy diet (see Section 1). Aspartate and GLU are absorbed from the intestinal lumen faster when they are free amino acids than when they are bound in peptides (Stegink, 1984b). Dietary carbohydrates stimulate ASP and GLU metabolism in the intestine and reduce the amount of ASP and GLU absorbed into the blood. Therefore, low carbohydrate 'diet' foods enable the maximum absorption of APM released ASP .

Aspartate and GLU are present in the brain in their metabolic role and as neurotransmitters, most probably as the transmitters released at excitatory synapses in the vast majority of cells in the mammalian CNS (Cotman and Monaghan, 1987; Cox and Bradford, 1978). Evidence for this includes (i) *in vitro* and *in vivo* demonstrations of Ca^{2+} dependent release of L-GLU and L-ASP; (ii) the identification of specific post-synaptic excitatory amino acid receptors responsible for mediating synaptic transmission; and (iii) the presence of fast inactivation mechanisms. Most CNS cells can be assumed to have receptors for ASP and GLU, irrespective of the presence of

other types of receptors on the same cell (Watkins, 1978). The action of ASP and GLU are mediated by at least four different receptors. Three of these have been identified using GLU analogues which have selective agonist action. These GLU-agonists are N-methly-D-aspartate (NMDA), kainate (KA) and quisqualate (QA). L-GLU displays a high affinity for the binding sites for each of these three agonists. The existence of the fourth receptor has been identified by a GLU analogue antagonist but a receptor has not yet been clearly identified (Cotman and Monaghan, 1987).

Among their many other roles in the brain, ASP and GLU are probably involved in the neurobiology of memory. Current neurobiological theories of memory emphasize a unique form of synaptic transmission which results in a long-lasting enhancement of synaptic efficiency (McNaughton, 1983). This process has been named long term potentiation (LTP). Most research on LTP has focussed on the hippocampal system (Lee, 1983), believed by most researchers to play an important role in memory (but see Jarrard, 1986). Aspartate and GLU appear to mediate transmission in some of the hippocampal pathways in which LTP has been experimentally induced (Wieraszko, 1983). Also, APV-5, a NMDA antagonist, has been found to block LTP formation in rats, *in vivo* and *in vitro*, impair spatial learning in the Morris water maze but not impair the the learning of a simple discrimination task in the Morris pool (Anderson, Baudry and Morris, 1985; Morris, Anderson, Lynch and Baudry, 1986). This finding supports the role of excitatory pathways in hippocampal LTP formation, as spatial memory, but not non-spatial memory, is believed to depend heavily on the workings of the hippocampus (Pribram, 1986).

Excitotoxicity of Aspartate

Despite these important metabolic and neurophysiological functions, high systemic doses of prenatally or postnatally administered ASP and GLU can destroy neurons in the circumventricular (CV) regions of the brain and the cingulate cortex of

neonatal mice (Olney, 1978). The most severely affected area is the arcuate nucleus of the hypothalamus which is involved in regulating neuroendocrine functions. High doses of ASP and GLU, by any route of administration, result in a well described syndrome of obese, stunted linear growth, sterility and some behavioural disturbances. Reported behavioural changes include delays in developmentally appropriate behaviours (Butcher and Vorhees, 1984), conflicting reports of increased and decreased activity levels (see Dawson and Lorden, 1981) and impaired performance on some learning tasks (see Section 1.5). Aspartate is as effective as GLU in inducing this syndrome (Pizzi, Tabor and Barnhart, 1978).

Olney (1978) demonstrated a correlation between the neuro-excitatory potential and the neurotoxic effects of a large range of GLU analogues. Specific antagonists of GLU or its analogues prevent these substances from exerting a neurotoxic effect (Olney, 1984). Neurotoxicity occurs when the excitatory action of these amino acids is not controlled. Normally, the ASP and GLU used in neurotransmission is synthesized within the neuron, released into the synapse in very small quantities and removed from the synapse by an extremely effective uptake system (Stone, Connick, Hastings and English, 1987). But if ASP and GLU are present outside the nerve cell for prolonged periods, it is claimed that they will "...literally excite the neuron to death" (Olney, 1987). As neurotoxicity results from the excitatory effects of these amino acids, they have become widely know as "excitotoxins". Different excitotoxins may exert their effects in slightly different ways (Coyle,1987; Schwarcz, Foster, French, Whetsell and Kohler, 1984), but it is generally believed that excitotoxicity can be either an immediate or a delayed event following the exposure of a neuron to an excitotoxin (Collins, 1986). Immediate excitotoxicity is caused when sustained depolarization of the neuron holds open membrane channels and allows an increase in ionic concentrations in the post-synaptic dendrites and soma. By osmosis, water also moves into the cell and causes an increase in tension on the cell membrane and, eventually, cell death. Sodium, calcium and chloride ions may be involved in the

intracellular increase in osmolarity (Collins, 1986, 1987; Schwarcz and Shoulson, 1987) but, in at least some brain cells, an influx of extracellular chloride ions is especially important (Rothman, 1985). Delayed excitotoxicity appears to rely more on the indirect action of calcium ions, but the exact mechanism of this process is not known (Collins, 1987).

Systemically administered excitatory amino acids are unable to reach most regions of the brain because of an efficient efflux carrier system for acidic amino acids in the blood-brain barrier (Pardridge, 1986). However, large doses of ASP and GLU have been shown to increase tissue concentrations in the CV and cingulate regions of the brain (Price, Olney, Lowry and Buchsbaum, 1981) as these regions are not protected by the blood-brain barrier. Other, extrahypothalamic regions of the brain are apparently unaffected, even by repeated high dose administrations of excitotoxins (Coyle, 1987). Systemically induced excitotoxicity is generally noted only when excitotoxins are administered at high doses so that plasma levels of GLU and/or ASP are elevated to about 60 - 100 µmol/dl in infant mice, which is about five to ten times normal levels (Applebaum, Daabees, Stegink and Kinkelstein, 1984; Stegink, 1984). In ten day old mice, the lowest effective experimental dose needed to produce these plasma levels and induce excitotoxicity is 0.5 g/kg administered orally and 0.35 g/Kg administered subcutaneously (Cotman and Monaghan, 1987). Adult animals require four times this dose to produce comparable neurotoxic effects (Olney, 1984), probably because of age related changes in absorption, metabolism and the rate of extra-cellular uptake by neurons and glial cells (Coyle, 1987).

The excitotoxic action of ASP, GLU and particularly their analogues is so effective at killing neurons that excitotoxins have been used extensively as research tools. Lesions induced by systemic administration have been used to investigate neurotransmitters, neural pathways and neurohormone function within the hypothalamus (Coyle, 1987). Regional injection of excitotoxins into the brain provide

a unique method of neurobiological investigation as excitotoxins damage only the dendrites and somata of neurons while sparing axons that pass through the region (McGreer, Olney and McGreer, 1978). This technique avoids the problem encountered with less selective lesion methods where the resulting behavioural disturbances may be due to damage caused by the lesion of axons passing through the area (Jarrard, 1986).

Of clinical interest, excitotoxic mechanisms are believed to be involved in a range of neurodegenerative disorders which involve memory impairment and display an overlapping pattern of selective neural degeneration. The neural degeneration associated with seizures, hypoxia, hypogylcemia, stokes and cardiac arrest is believed to caused by a sudden release of ASP and GLU from neurons while uptake mechanisms are temporarily inoperative (Collins, 1987; Colye, 1987). Indeed, NMDA antagonists have been shown to protect brain tissue from this kind of damage (Schwarcz *et al.* , 1984). Progressive degenerative disorders believed to involve excitotoxic mechanisms include Huntingtons disease (Schwarcz and Shoulson, 1987) and Alzheimers disease (Deutsch and Morihisa, 1988). The 'leakage' in the blood-brain barrier observed in normal elderly individuals and individuals with Alzheimers disease may allow excitotoxic doses of ASP and GLU to reach, and damage, sensitive regions of the brain.

Neurons in the hippocampal system appear particularly sensitive to excitotoxic damage and from a range of other metabolic and toxic insults (Walsh and Chrobak, 1987). This damage to the hippocampus is probably a major contributing factor to the memory disturbances which accompany some of the disorders discussed in the previous paragraph (but not Huntingtons disease). Although the blood-brain barrier protects the hippocampal formation from increased levels of excitatory amino acids, some hippocampal neurons are particularly vulnerable to intraventricular injections of KA (Nadler, Perry and Cotman, 1978). Also, the cingulate cortex is unprotected by the

blood-brain barrier and is a target for efferent projections arising from the CA1 and CA3 cell fields in the hippocampus (see Jarrard, 1986). As excitotoxic lesions to afferent and efferent projections of the hippocampus impair memory function in rats (Jarrard, 1986), excitotoxic amino acids may be able to exert an indirect effect on the hippocampal system by acting on the cingulate cortex.

Risks from Aspartame Released Aspartate

It is unclear whether humans are likely to be affected by the excitotoxic action of ASP and GLU. Olney and colleagues have reported neuronal necrosis in infant non-human primates given large doses of GLU (Olney and Sharp, 1969; see Olney, 1984) but four other research groups were unable to detect such lesions, even when using large doses of APM and MSG which produced gross elevations in plasma ASP and GLU levels (see Stegink, 1984a). But whether or not APM-induced excitotoxicity is a danger to humans, sub-toxic doses of ASP and GLU may exert other more subtle effects. Olney and colleagues (see Olney, 1984; Olney and Price, 1978) reported that sub-cutaneous doses of 100 mg/Kg GLU given to infant mice induced neuroendocrine changes which were probably secondary to excitatory stimulation of neurons in CV regions which regulate pituitary function. Sub toxic doses of NMDA have produced similar findings in both rats and monkeys (Wilson and Knobil, 1982). Olney has suggested that neurohormonal changes resulting from sub-toxic doses of excitatory amino acids may lead to the slow development of "silent lesions" (Olney, 1984). Efforts to replicate these neurohormonal finding have been somewhat mixed and some authors have attributed Olney's findings to circadian variations in hormonal levels (e.g., Sturtevant, 1984). Nevertheless, some researchers (e.g., Dawson, 1987) remain concerned about possible APM induced disturbances on the neuroendocrine system, especially in the young.

While the rate and efficiency of the gastrointestinal absorption of APM released ASP and PHE have not been well studied, Olney (1984) notes that the literature on MSG shows that humans absorb GLU probably ASP much more efficiently than other species. However, Stegink (1984b) reported that plasma ASP levels in humans did not rise significantly following 34 mg/Kg APM with or without accompanying carbohydrate. Pardridge (1986) used data from MSG studies to estimate that plasma ASP levels following an acute dose of 50 to 100 mg/Kg APM, without carbohydrates, would rise modestly to about 10 μmol/dl. However, Olney notes that the literature relating to GLU absorption suggests that there is a large variation in the rate at which humans absorb GLU and the data reviewed by Olney shows that plasma levels of GLU increase at a hyperbolic rate with GLU ingestion. Hence significant increases in ASP levels may be achieved by heavy consumption of APM in at least some individuals.

Groups Vulnerable to the Effect of Aspartame Released Aspartate

The discussion in the previous paragraphs revealed that it is unclear whether systemically administered excitatory amino acids can exert excitotoxic or neurohormonal effects of humans or whether APM released ASP can significantly increase plasma ASP levels. Also, the blood brain barrier will isolate the brain from any changes in plasma ASP level (see page 15). However, if APM released ASP can enter the body, those individuals with a degenerating or incomplete blood-brain barrier may be especially at risk. Indeed, the 'leakage' in the blood brain barrier noted in elderly people and individuals with Alzheimers disease has been suggested as a way that excitatory amino acids may enter the brain and exert a excitotoxic neurodegenerative effect (Deutsch and Morihisa, 1988). APM related increases in plasma ASP may be an extra hazard for this group. Another group at risk from APM released ASP may be the unborn and neonates. The blood-brain barrier does not become functionally mature until shortly after birth (Price *et al.* , 1981) and hence the

unprotected prenatal brain could be exposed to the adverse effects of systemically administered excitotoxins. However, to reach the fetus, any substance must first cross the placental barrier. Unlike most amino acids, ASP and GLU are not concentrated in the fetus by the placenta. Instead, fetal plasma levels have been experimentally elevated only when maternal plasma concentrations are 70 times normal levels of GLU (Garattini, 1979) and over 100 times normal levels of ASP (Stegink *et al.*, 1979).

Studies which have investigated the placental transfer of ASP and GLU have done so during the late stages of gestation, or else they have not stated the stage of gestation at which they were performed. While investigation of only these late stages may be methodologically easier, it overlooks the rapid development of the placenta which occurs mainly during the first third of mammalian gestation. The initial structure of the yolk sac placenta gives rise to the allantois and the chorion which eventually fuse to form the mature, chorioallantoic placenta (Mossman, 1987). The transport properties of these changing placental structures varies with their development (Ullberg, Dencker and Danielsson, 1982) but not enough is known about these changes to accurately predict the transport properties at one stage of development from knowledge of the transport properties at another stage.

The chorioallantoic placenta is developed in humans by the third month of gestation (Thomas, 1985) and in rats by the twelfth day (Juchau, 1982). While the mature placenta appears to be an effective barrier to high maternal plasma levels of ASP, the developing placenta may not be so effective. The nervous system, which is the first organ to develop in the embryo (Patel, 1987), is especially sensitive to the effects of teratogenic agents during the period of organogenesis (Vorhees, 1986b). Organogenesis occurs from days 13 to 56 in humans (Thomas, 1985) and days 6 to 16 in rats (Vorhees, 1986). Thus, there is considerable overlap between the periods when the embryo is most sensitive to teratogenic insult and the placenta is least developed. If ASP can enter the fetus prior the the functional maturity of the placenta, it may

exert an effect on the developing nervous system through it's excitotoxic action or through it's normal neuroactive properties. The unusual presence of neuroactive substances, including unusual concentrations of neurotransmitters, can disturb the development of the nervous system by changing the balance and timing of the organization of developing neuronal interconnections (Patel, 1987).

To conclude this discussion of the risks associated with APM released ASP, it must be emphasised that the hypothesised danger rests heavily on a number of uncertainties which exist in the literature. For instance, it is not clear that the ASP released from APM can significantly alter plasma ASP levels, cross the immature placental barrier or exert a teratogenic effect. However, it is clear that the widespread use of APM warrants the thorough invesigation of all these possibilities.

1.3.4. Aspartame and Related Peptides

While the metabolites of APM, namely ASP, PHE and methanol, are present in the normal diet, the intact APM peptide and other peptides produced by APM's metabolism and degradation are synthetic substances which have no natural occurrence or function. These synthetic substances may be potentially harmful. Aspartylphenylalanine is a peptide released during the intestinal hydrolysis of APM. There are a number of other peptides produced by the normal slow degradation of APM and the rate of this degradation can be greatly increased by changes in pH and temperature (Pardridge, 1986). Little is known about most of these decomposition products (Bada, 1987). One long term feeding study found no toxic effects when the diketopiperazine derivative of APM was added to the normal diet of rats (Ishii, Koshimizu, Usami and Fujimoto, 1981) but the crystals of the substance added to the diet were sufficiently coarse that the rats may have avoided eating them (see Olney, 1987).

Intestinal metabolism protects the body from the potentially toxic effects of large quantities of peptides in the blood (Mathews, 1984) and the fast intestinal hydrolysis is presumed to prevent APM from entering the blood (e.g., Oppermann, 1984). However, some of the known side effects of APM ingestion, such as skin reactions, are unlikely to be caused by APM's natural metabolites and therefore they are probably caused by an APM or related peptide (Pardridge, 1986). This suggests that APM peptides can enter the blood of at least some, if not all, people.

The presence of synthetic peptides in the maternal system may be an extra hazard for the fetus. A major role of the placenta is to prevent unwanted substances from entering the fetus but the placenta may not be very effective at regulating the transport of synthetic substances which have not been present in the environment during the placenta's evolution (Ullberg, Dencker and Danielson, 1982). The lack of effective regulation of the placental transfer of synthetic substances may be why so many drugs, pesticides and industrial chemicals can exert teratogenic effects on the fetus.

1.4. Behavioural Toxicology of Aspartame

The previous sections have identified ways that APM may be harmful to the organism. While most investigations of the toxicity of an agent focus on the pharmacology of it's action, toxic agents also have behavioural effects. It is this behavioural toxicology that is of most interest and most relevance to psychology. While it it has been claimed that APM is the most thoroughly studied food additive ever (e.g., Stegink and Filer, 1984; Gossel, 1984), there appear to be surprisingly few studies on the behavioural effects of APM. In an effort to locate behavioural studies, the present author carried out an on-line computer search of psychology and medical databases, searched sources cited in a book chapter on the behavioural effects of APM

(Butcher and Vorhees, 1984), searched for APM related titles listed in "Current Contents", and consulted a list of all APM related literature published in the world between 1981 and late 1987 (published in the 1987 American Congressional Hearing on Nutrasweet). While some behavioural studies were probably submitted to the FDA as part of the petition for the approval of APM, these are not freely available for review. Excluding studies which examined only activity measures associated with the excitotoxic syndrome (Section 1.3.3), only five studies were found which examined the behavioural toxicology of APM. This section will review these studies.

Potts, Bloss and Nutting (1980) added to the normal diet of weanling rats 0, 5.5 or 11 g/Kg APM, or PHE doses equivalent to the amount of PHE consumed in the APM conditions. The rats were fed this diet for 90 days. Males, but not females, in the high PHE and APM groups made significantly fewer avoidance responses in a shuttle-box avoidance task. The high PHE male group also received significantly more shocks during the second half of testing on a Sidman (non-discriminated) avoidance task, but not during the test overall. There were no significant dose dependent differences between any groups on cage activity measures. The authors attributed the observed effects of APM to it's PHE constituent.

Reynolds, Bauman, Stegink, Filer and Naidu (1984) fed five groups of four infant stumptail monkeys infant formula diets containing 0, 1, 2, or 3 g/Kg APM or 1.65 g/Kg PHE for 270 days. They found no difference the groups on a range of developmental measures and the only distinguishable difference was elevated water intake in the 3 g/Kg APM and 1.5 g/Kg PHE groups. Suomi (1984) tested the same animals on a standardized primate learning test battery and reported no differences in performance between the experimental groups, control group or norms gathered from previous studies that used the same test battery. While large group sizes are not feasible in primate studies differences must be large to achieve statistical significance, the

authors do not mention any indication of groups differences but neither do they report the actual data.

Nomura (1985) administered a single acute dose of 250 mg/Kg APM by stomach tube to a group of rats that had previously been trained on (i) a brightness discrimination task with a multiple variable interval and extinction schedule and (ii) a fixed ratio (50) scehdule. Relative to controls, the APM group displayed no difference in patterns of responding on these schedules.

Gershbein, Dachman, Gershbein and Kambhampati (1987) examined exploratory behaviour using the hole-board test with rats administered low doses of APM by drinker. They found that males, but not females, administered 0.0625% by drinker for 42 days from 46 days of age made significantly fewer hole pokes in later minutes of the trials than control rats. However they were unable to replicate this finding. In a separate experiment they found that weanling rats given 0.625% APM for 19 days and then 0.0625% APM for 24 days made significantly more hole pokes than control rats or rats which received 0.0625% APM throughout the 45 day period. However, these workers were unable to observe any differences between rats given the spit 0.625/0.0625% APM dose regime from 47 days of age. The weight quantity of APM consumed by the rats was not reported in this study. Gershbein *et al.* did not try to interpret these conflicting findings.

Pinto and Maher (1988) found that pretreating rats by oral intubation with 1 to 2 g/Kg APM significantly increased the sensitivity of mice to chemically induced seizures and decreased the time between when the pro-convulsant agent was administered and when seizure was first observed. They also found that equimolar amounts of PHE, but not ASP or methanol, induced a similar sensitivity to seizure and the co-administration of APM and valine, a large neutral amino acid, did not increase seizures. They concluded that the sensitivity to seizures was mediated by PHE,

possibly secondary to a suppression of brain catecholamine synthesis caused by PHE lowering the brain uptake of tyrosine (see Section 1.3.2).

Together, these five studies tentatively suggest that APM may exert an adverse effect on behaviour at high doses and possibly at low doses. Both the Potts *et al.* (1980) study and the Gershbein *et al.* (1987) study report an unclear pattern of sex differences and significant findings on only the second half of some of the tests. These few studies have examined different aspects of behaviour and, as a result, no behaviours have been thoroughly studied. Clearly, the behavioural toxicology of APM has not been adequately investigated.

1.5. Behavioural Teratology of Aspartame

While toxicology focuses on the effects of postnatal exposure to an agent, teratology is primarily concerned with the morphological and functional effects of prenatal experiences. This pre/post-natal dichotomy overlooks the continuing process of development and the different developmental stage of different species at birth. Instead, it places emphasis on the time of dramatic change in the nature of the offspring's reliance on the mother (Vorhees, 1986a). Teratology originally developed from toxicology with a particular concern for the study of congenital birth deformities, but it has expanded, or perhaps a sub-specialty has emerged, with an emphasis on the behavioural abnormalities which arise from the prenatal exposure to a teratogen (Grimm, 1987). These behavioural differences are assumed to reflect some level of morphological change, but the behavioural changes are considered to be more sensitive measures of teratogenicity than physical malformations as the ability of tissue to perform its adaptive function is considered to be a more valid measure of its integrity (Butcher and Vorhees, 1984).

After briefly mentioning the importance of behavioural studies in it's introduction, the fifth edition of the Catalogue of Teratogenic Agents (Shepard, 1986) considered APM not to be teratogenic on the basis of two studies by the same research group which reported no significant increases in plasma amino acid levels after APM ingestion. However, the evidence outlined in earlier sections suggests that the fetus whose mother ingests APM may in fact be at risk from the effects of PHE and/or ASP and possibly from the effects of the intact APM peptide. Behavioural measures may offer a sensitive index of subtle effects but there have been few studies of the behavioural teratology of APM - the current author managed to find only two behavioural teratology studies with APM. These studies are reviewed below. Olney (1987) mentions that two teratology studies were submitted to the FDA by G.D. Searle and Co. but these may not have been behavioural studies, these studies are not published and, according to Olney, they are of dubious reliability.

Mahalik and Gautieri (1984) administered 0, 1 or 4 g/Kg/day APM, or saline control solution, by stomach intubation to pregnant mice during days 15 to 18 of gestation. While APM had no effect of the development of three reflexes studied, namely negative geotaxis, surface and air righting, there was a significant dose-dependent delay in the age at which the APM treated offspring developed a visual placing reflex, defined as the rat extending it's front paws to a rope placed below it. The authors concluded that this delay in such a basic developmental task suggested that prenatal exposure to high doses of APM can cause functional impairments in mice. However, this study did not employ a cross-fostering procedure and therefore the observed differences may have been due to the effects of APM on the dams.

Brunner, Vorhees, Kinney and Butcher (1979) added 0, 2, 4 or 6 % APM or 3 % PHE to the normal diet of groups of male and female Sprague-Dawley rats from before 14 days before mating. The PHE group was included to provide data to compare with any APM treatment effects. The dams continued this diet until their offspring were

weaned and the offspring continued these diets throughout their life. The dose of APM consumed by the 6% APM dams was about 5 g/Kg/day throughout gestation and about 9.5 g/Kg/day throughout lactation. Post-weaning, the 6% APM offspring consumed about 9 g/Kg/day APM. The lower dose groups consumed approximately proportional doses of APM, with respect to their experimental dose condition (e.g., 0, 2, or 4% APM).

The 6% APM and especially the 3% PHE groups showed a significant increase in offspring mortality and significantly delayed development. The 4% APM group showed some delays in development. When tested pre-weaning in an open field test, the 6% APM and 3% PHE groups showed longer initial latencies to leave the centre square and reduced locomotor activity. When tested post-weaning in an open field test, only the female 6% APM group showed an increased initial latency.

The offspring in Brunner *et al.* 's (1979) study were weaned at 21 days and housed in individual cages at 24 days. Post weaning, all animals were tested on a running wheel and a rotor rod balancing test, and two learning tasks. The learning tasks were (i) an active avoidance task, with a wheel turn response and white noise signalling foot-shock; and (ii) a two-chamber (light/dark) passive avoidance task, with foot-shock in the dark chamber on the first day. No treatment effects were observed on these learning tasks. However, the generality of these results is seriously compromised as the offspring were reared in social isolation. Compared with group-housed litter-mates, rats reared in social isolation are well known to exhibit enduring changes in their performance on a variety of activity and learning tasks (e.g., Dalrymple-Alford and Benton, 1984; Einon, 1980). It is possible that an isolation by treatment interaction may have obscured any effects.

The Brunner *et al.* (1979) study also examined two additional groups of offspring (0 and 6 % APM) under identical conditions as above. However, at birth these offspring were cross-fostered to the dams of the other condition (i.e., pups of control dams were

given to 6% APM dams, and *vice versa*). The offspring which received APM postnatally showed similar developmental delays as the 6% APM and 3% PHE groups in the main experiment, but the offspring which received APM prenatally showed no such impairments. From this experiment, the authors concluded that the adverse effects observed in the main experiment were due to exposure to APM during the postnatal period and, therefore, the effects are not actually teratological effects. In a later article, the same authors claimed the effects were probably due to the inability of the young animal to effectively hydrolyse PHE (Butcher and Vorhees, 1984). Their conclusion seems unwarranted for the following reasons: The authors (Brunner *et al.*, 1979) reported that in the cross-fostering experiment the APM fed dams and control dams were similar weights at the time of birth but APM fed dams lost weight throughout lactation, relative to controls. Also, at birth there was no difference between the weights of pups in either group, but pups that were fed by APM fed dams lost weight throughout lactation, relative to controls. These combined findings suggest that the high doses of APM affected the lactating rat. It would be more parsimonious to conclude that the effect on the dams may have in turn affected the pups by altering the standard of nutrition and/or care which they received. To determine whether any of the observed effects were due to prenatal or postnatal exposure to APM or maternal factors, both control and APM exposed offspring would need to be fostered to control dams.

Despite the uncertainty over the actual origins of the effects in the Brunner *et al.* (1979) study, the authors concluded that the effects were due to the PHE moiety of APM. Similar explanations have been made in behavioural toxicology studies (e.g., Potts *et al.*, 1980). However, at least two studies have reported altered learning performance and changes in activity measures following prenatal exposure to MSG. The functional similarity between ASP and GLU suggest that similar doses of ASP may exert similar effects. These two studies will be briefly reviewed.

Butcher and Vorhees (1984) cite an earlier study published by their group which found that 5.1% MSG administered in the diet of pregnant rats produced developmental delays, slower (impaired) active avoidance and longer 48-hr passive avoidance response latencies. This increase in passive avoidance latency could be interpreted as increased retention and enhanced memory, but performance on passive avoidance tasks can be confounded by emotional factors. This study could not be found in the reference cited and therefore the actual MSG dose and test procedures could not be established. In the second study, Frieder and Grimm (1984) administered 10 g/Kg/day MSG in the drinking water of three female rats throughout the last two thirds of their pregnancy. Two untreated dams produced control condition offspring. (It is assumed that offspring were not cross-fostered as a cross-fostering procedure was not reported). There were no significant differences between the performance of the groups on a shuttle-box avoidance learning task or a simple successive brightness discrimination task (black/white runway). However, rats prenatally exposed to MSG made significantly more errors in the later trials of a six unit simultaneous discrimination learning task. The MSG rats also exhibited morphological and activity level changes attributable to the excitotoxic syndrome (Section 1.3.3). These two behavioural teratology studies with MSG support the idea that APM released ASP may be capable of exerting a behavioural teratogenic effect.

1.6. Rationale for the Present Study

The above discussion suggests that APM may be able to exert adverse effects on an animal. These effects may result from both the PHE and ASP moieties of APM and perhaps the intact APM peptide. The prenatal animal may be most vulnerable to these effects.

It was proposed above that ASP may be able to enter the maternal system and the embryo during the early period of gestation when the placenta has not reached a level of functional maturity. As ASP is a putative neurotransmitter, it may affect the embryo by being present in the developing nervous system at unusual concentrations during the initial stages of organogenisis and neural proliferation. Aspartate may also affect the young nervous system through its excitatory and/or excitotoxic action. Aspartate is involved in the neurobiology of memory and excitatory pathways are involved in LTP formation in the hippocampus. As the hippocampal system is unusually vulnerable to excitotoxic, toxic and metabolic insults, prenatal exposure to low dose APM released ASP may exert a subtle behavioural teratogenic effect on the learning and memory ability of the animal. Also, PHE is concentrated in the prenatal brain and can impair learning and memory performance, most probably in linear dose-dependent way. Therefore low doses of APM may be able to exert a behavioural teratogenic effect that may be detectable by examining learning and memory performance. The present study sought to test this hypothesis by examining the performance of rats, prenatally exposed to two low doses of APM, on a complex learning and memory task. In light of the sex differences noted in the studies by Brunner *et al.* (1979), Potts *et al.* (1980) and Gershbein *et al.* (1987), the present study examined both male and female offspring.

With respect to the teratological nature of the present study, a cross-fostering procedure was employed. An agent that appears to exert a behavioural teratogenic effect may do so indirectly by changing the rearing habits of the mother or changing the standard of nutrition and/or care which she supplies to her offspring. Cross-fostering all offspring to a control group, non-biological parent allows treatment effects to be determined by eliminating the possible indirect effects of the treatment on the dams. Vorhees (1986b) claims that cross fostering is a resource intensive procedure that is necessary only in the later studies of the investigation of a teratogen. He considers preliminary experiments, without cross-fostering procedures, must first establish evidence of apparent behavioural teratogenic effects. While this may be an acceptable strategy for extensive programmes of behavioural teratology research, it is not suited to the single experiment, such as the present study. It is noteworthy that the inadequate cross-fostering procedures employed by Vorhees and associates (Brunner *et al.*, 1979, see Section 1.5) undermined the only previous behavioural teratology study of APM which has investigated performance on learning tasks.

The two previous behavioural teratology studies of APM (Mahalik and Gautieri, 1984; Brunner *et al.*, 1979), both used high doses. Vorhees (1986) recommends that behavioural teratology investigations begin with large doses and only use lower doses once a risk has been established. However, the literature on APM demonstrates that high dose studies are not always followed by low dose studies (e.g., Brunner *et al.*, 1979) and high dose studies tell us little about the effects of doses and patterns of use which more analogous to human consumption. The use of a low dose of APM is supported by the initial findings of the Gershbein *et al.* (1987), that 0.0625% APM by drinker may affect exploratory behaviour in rats, and recent calls in the literature to investigate the effects of APM at doses more relevant to human patterns of consumption (e.g., Pardridge, 1986; Elsas, 1987; Metzenbaum, 1987). Hence, the present study chose to investigate the effects of prenatal exposure to low doses of APM . Two low doses were used to enhance the likelyhood of finding an effect.

The present study used a radial arm maze task as a complex learning and memory test and the Hughes exploration box as a measure of exploratory behaviour. There are many different experimental procedures used to measure learning and memory in the laboratory rat, but in commenting on a series of behavioural teratology studies, Grimm and co-workers (see Grimm, 1987) noted that behavioural deficits were not observed on simple avoidance tasks where as they were observed on more complex simultaneous discrimination learning tasks, some of which involved avoidance responses. Many of the studies reviewed above employed active and passive avoidance tasks to measure learning and these tasks have been used together as measures of hippocampal damage (Rodier, 1978). However, there are a number of problems with these tasks (Tadokoro, 1987; Gerber and O'Scaughnessy, 1986). These procedures require an animal to either perform a particular behaviour (active) or not to perform a particular behaviour (passive) in order to avoid the presentation of aversive stimuli. As well as differences in memory, avoidance behaviour is also affected by differences in the sensitivity and reactivity of the animal to aversive stimuli. Hence differences on avoidance tasks may reflect sensory and emotional differences in the animals. Also, the behavioural effect noted on these tasks can be critically dependent on the actual task performed. For example, some patterns of hippocampal damage can decrease avoidance on a one way active avoidance task but increased avoidance on a two way active avoidance task; performance of passive avoidance tasks can be similarly mixed (Gray and McNaughton, 1983).

The present study used a radial arm maze to test the learning abilities of rats prenatally exposed to a low dose of APM from conception. The radial arm maze (RAM), also known as the Olton spatial maze (Adams, 1986), is composed of a central platform with usually eight or more arms radiating from it like spokes of a wheel (Olton, 1987). A guillotine door is positioned at the entrance of each arm to so the rat (or mouse) can be prevented from entering that arm. There is a barrier on one side of

each arm near the centre of the maze which prevents the rat from jumping from one arm to the next without revisiting the hub. While exact test procedures differ, the rat is usually confined to the centre of the maze and then allowed to search the maze for food placed in the food wells at the end of some or all arms. The guillotine doors are lowered each time the rat enters an arm and then raised shortly after the rat has returned to the centre of the maze. This procedure ensures that the next arm which the rat chooses is determined by at least some amount of choice and not by the response habits of the animal (Olton, 1983a).

The RAM is a well validated measure of spatial memory in the rat. The number of times that a rat visits or revisits an arm which contains no food is taken to be an index of spatial memory. The RAM is claimed to have ethological validity as it can be conceptualized as representing a foraging task (Olton, 1987). In the RAM, the rat is required to learn a list of spatial locations. List learning is also a feature of human cognitive tests, such as the Weschler scales, and performance on list learning tasks is usually impaired in humans suffering from amnesia. Impaired RAM performance is found in rats after experimental brain damage to the septo-hippocampal system and damage to these regions of the brain in humans is believed to result in selective memory impairment, especially in episodic memory (Squires, 1983). The RAM gains psychological validity from observations of similarities between the memory effects seen in humans, such as primacy and recency effects and proactive interference, and the behaviour of rats in the RAM (see Olton, 1987).

Because of the above features, the RAM has been used extensively to investigate the psychological and neurobiological processes in memory. It has been used as a tool in behavioural toxicology (Walsh and Chrobak, 1987), behavioural teratology (Adams, 1986) and neurobiology (Jarrard, 1986). While not sensitive to every kind of neural insult, RAM performance displays an acknowledged sensitivity to lesions of the hippocampus and its afferent and efferent projections (Walsh and Chrobak, 1987;

Jarrard, 1986). Standard RAM procedures employ positive reinforcement and thus avoid the problems with emotional variability that confound the interpretation of avoidance tasks.

The RAM represents a complex test of learning and memory. Complex tests increase the chance of detecting performance differences, especially subtle differences in cognition that may not show up on less demanding tasks (Olton, 1983b). In order to make the RAM task a sufficiently demanding task, the present study used a 12 arm RAM with six of the arms baited and six of the arms unbaited. The baited and unbaited procedure used in study were first employed by Olton and Papas, (1979) to distinguish between two theoreticaly different types of memory: working memory and reference memory. Working memory has been described as the memory of information which is pertinent to only one instance or trial, whereas reference memory is a more general type of memory which is relevant to a large number of similar instances or trials (Olton, 1983b; Morris, 1983). These constructs are not considered to be completely separable as working memory tasks (where is the food?) always require the performance of some behaviour which requires reference memory (run to the end of the arm to find the food). Olton and Papas proposed that ideal performance in the RAM with baited and unbaited arms requires the both working and reference memory. The rat requires a working memory of events in the current trial to determine whether it has already been to a baited arm. Reference memory is needed to learn the general rule that that no matter what trial the rat is in and no matter what the rat has already done in the present trial, it will not find food on the first entry and subsequent re-entries to *some* arms. However these operational definitions of working and reference memory are not universally accepted as Jarrard (1986) considers that remembering not to re-enter an unbaited arm within a trial is a working memory task. To avoid this unresolved issue, the present study will refer to memory errors by their operational definitions: re-entries to baited arm, initial entries to unbaited arm and re-entries to baited arms.

As well as learning, behavioural teratology studies frequently sample a variety of types of behaviour, including sensory functions, motor abilities, measures of development, activity, emotional behaviour and exploratory behaviour (see Adams, 1986; Rodier, 1978). The present study chose to examine the effects of prenatal exposure to APM on exploratory behaviour, and in so doing possibly to extend the tentative findings of Gershbein *et al.* (1987), that low dose postnatal exposure to APM reduced exploratory behaviour.

Gershbein *et al.* (1987) used hole-poking on a hole-board test as a measure of exploratory behaviour, even though they considered the reliability of this test as a measure of exploration had not been established. Hole-poking in a novel environment is believed to measure exploration as change in the environment is presumed to increase exploratory behaviour and one form of exploratory behaviour in the rat is a tendency to dip its head into a dark hole (Mackintosh, Chance and Silverman, 1977). But as the animal is forced into the novel environment, what is measured is the rat's behaviour in the presence of inescapable novelty and not it's novelty seeking behaviour (Robbins, 1977). The hole-poking task tries to separate whole body locomotion from discrete head dipping behaviour, but hole poking scores are highly influenced by the activity level of the animal (Robbins, 1977). Studies with amphetamine have shown that hole-poking behaviour can vary somewhat independently of activity level (File, 1978) but the level of activity of the animal remains a serious uncontrolled and unmeasured confounding factor. Indeed, the hole poke test has been used as a measure of activity only (Adams, 1986).

The present study measured exploratory behaviour in the Hughes exploration box. This procedure overcomes many of the the limitations of the hole poke test. The Hughes exploration box consists of a Perspex box divided into two separate halves, each half being sub-divided into two cells. The animal is familiarized with one half

of the box and then, after a suitable habituation period, it is allowed access to the other, novel side of the box. As exploration always depends on the animal being active, the Hughes exploration box allows the simultaneous and independent measurement of exploratory behaviour and locomotor activity. A time sampling technique determines the amount of time spent in the familiar and the novel sides of the box and the percentage of time spent in the novel side of the box provides a measure of exploratory behaviour. A measure of locomotor activity is provided by a record of the total number of cells entered. The independence of the measures of activity and exploration have been confirmed by observations of increased, decreased and unchanged exploration with each of increased, decreased and unchanged activity (see Hughes and Pither, 1987).

Changes in exploration are inferred only when (i) the activity measure remains constant while the exploration measure changes, (ii) the activity measure changes while the exploration measure remains constant, or (iii) both the exploration measure and the activity measure vary but in opposite directions. Thus, the Hughes exploration box is less affected than the hole-poke test by the problem of activity confounding the measure of exploratory behaviour. Also, the Hughes exploration box provides a true measure of novelty seeking behaviour by allowing the rat to choose between the familiar and the novel sides of the box.

Chapter 2

Experiment

2.1. Methods

2.1.1. Subjects

The present study used randomly derived albino, Wistar rats from the University of Canterbury Psychology Department colony. When the females were at estrus, as determined by regular vaginal examination, fourteen pairs of male and female rats, aged 126-146 days, were placed together in separate plastic cages, 46 x 29 x 16.5 cm high, with stainless steel lids. After mating was observed, the males were removed and the dams were randomly assigned to one of three groups. From day one of pregnancy until parturition, two groups were administered APM dissolved in their drinking water at concentrations of 0.05% (n=4 dams) or 0.10% (n=4 dams) by weight. The solutions were buffered with citric acid to a pH of 4.3 ± 0.2 as APM is maximally soluble and degrades very slowly at this pH (Reynolds, 1982; Gossel, 1984). The drinking water of the third, control group (n=6 dams) was buffered to the same pH. All dams received these solutions *ad. libitum* throughout gestation and until parturition. Every three days, the body weight of the dams and the weight of the solution was recorded and all three solutions were changed. Drinking solutions were made up fresh every 6 days and stored at 4 °C . The APM used in the present study was kindly supplied by Sutton Distributors, Auckland, New Zealand. The APM was found to be pure when examined by nuclear magnetic resonance spectroscopy in the Chemistry Department of the University of Canterbury, Christchurch, New Zealand.

From the body weights and solution weights, the dose of APM consumed was calculated for the dams from which the experimental subjects were taken. Across days, the 0.05% APM dams consumed an average of 60.3 ± 6.2 mg/Kg/day (mean ± S.D.) of APM and the 0.10% APM dams consumed an average of 123.6 ± 13.03 mg/Kg/day of APM.

The experimental subjects were born at 20-23 days after mating and litter sizes ranged from 9 to 14 pups. At birth, experimental solutions were removed and fresh drinking water was supplied to all groups. Within 24 hours of birth, all litters used in the experiment were cross-fostered to control group dams that has also delivered a litter within 24 hours. If a suitable control dam was not available to accept a litter within this 24 hr period, the litter was not used in the experiment.

Intraspecific genetic variation can cause behavioural differences between members of the same species and this genetically determined behavioural variation can be a confounding factor in behavioural teratology studies. To control for this genetic effect, behavioural teratology studies increase the genetic variation of experimental groups by taking few, or ideally only one, subject from each experimental litter. Controlling genetic variation is a resource intensive requirement and a balance must be found where this effect is adequately balanced without an excessive use of resources. In the present study, it was decided that these demands could be adequately met by taking three offspring form each of three experimental litters. Of the original 14 dams, five (two control dams, two 0.05% APM dams and one 0.10% APM dam) did not produce successful litters and two of the control litters were not used due to a lack of suitable cross-foster mother within 24 hours of birth. One 0.05% APM dam gave birth to seven female, but no male, pups. Table 2.1 (p. 38) shows the size and genetic composition of the groups. The male and female control and 0.10% APM groups were each made up of three rats from each of three litters. However, the nine 0.05% APM female rats came from two litters and the six 0.05% APM males came from only one litter.

Table 2.1
Summary of Experimental Group Size, Number of Litters in the Groups and the Quantity of Aspartame Received by Dams

Group	Dose APM mean ± S.D. (mg/Kg/day)	Number of Subjects per group		Number of Litters in group (rats per litter)	
		Males	Females	Males	Females
Control	0.0 ± 0	9	9	3 (3,3,3)	3 (3,3,3)
0.05% APM	60.3 ± 6.3	6	9	1 (6)	2 (4,5)
0.10% APM	123.6 ± 13.0	9	9	3 (3,3,3)	3 (3,3,3)

Subjects were weaned at 25-28 days and group housed in plastic cages, 46 x 29 x 16.5 cm high, with stainless steel mesh lids. Each cage contained three same-sex rats from the same experimental group and, where possible, the rats were from different litters. A record was kept of the litters which the animals came from so that the experimental results could be analysed for the presence of litter effects. The rats were housed in a room maintained at an ambient temperature of 24 ± 1 °C on a reversed 12-hr light-dark cycle with lights off 6 am to 6 pm. Before the RAM testing began, the reversed light dark cycle was adjusted to lights off 12 pm to 12 am to accommodate the availability of the experimenter.

Standard dry rat food and drinking water were available *ad libitum* from weaning until after the Hughes exploration box testing. The RAM procedure requires the body weight of the rats to be reduced to a percentage of their free feeding weight. As the rats in the present study were young and still growing rapidly, their body weight was maintained at 80 to 85 percent of their growth-adjusted expected body weight during testing in the RAM. The expected body weight figure was calculated for each sex from the body weights of three male or three female control group rats which were allowed *ad. libitum* access to food throughout the experiment. During the weight reduction and pretraining phase, all rats were weighted daily and the quantity of food that was required to maintain the growth adjusted weight of the rats was determined. During the RAM testing, the rats were fed the predetermined quantity of

food daily and were weighed, on average, every three days. Any below-weight rats, relative to cage-mates, were allowed additional food in a separate cage.

2.1.2. Apparatus

Hughes Exploration Box

This test simultaneously employed four identical exploration boxes. Each box was 40x40 cm square, 20 cm high and constructed of Perspex sides, two half-sized Perspex hinged lids and four quarter-sized galvanized metal floor plates. Each box was divided into four equal sized quadrants by opaque Perspex walls. Each wall had a 7.5x20 cm opening centered at the middle of the quadrant, which allowed access between adjacent quadrants. Two opaque guillotine doors could be lowered from above to block two of the openings and divide the box into left and right sides. Each box was enclosed in a sound proof ventilated chamber with an internal volume of 250 litres. The ventilator fan provided a background masking noise in the box of 80-85 dB (Dawe Sound Level Meter, Type No.1400E, weighting 'C'). Each box was illuminated by a centrally mounted 8 W fluorescent tube which provided a light intensity, close to the centre of the box, of 600 lux ± 5% (Toshiba SP1-5 Photocell Illuminometer). Observation of the rats were made through a small one way window in the front wall of each chamber with the door of the chamber closed.

Radial Arm Maze

The central platform of the maze was a wooden dodecahedron of 38 cm diameter, painted black and raised 65 cm above the floor. The 12 unpainted aluminium arms (65.5 x 8.5 cm) radiated out from the 12 faces of the central platform. Each arm had 3.5 cm high sidewalls, and a 23cm high perspex wall along the central 20 cm of one side to ensure that the rat returned to the centre of the maze before choosing another arm.

There was a recessed food well drilled in a black painted wooden block (5 x 8.5 x 1 cm) at the end of each arm. Access to the arms was governed by 7cm wide transparent Perspex guillotine doors mounted on a 38 cm high wooden frame that was attached to corners of the central platform. The doors could be remotely raised or lowered by a system of overhead strings.

The 12-arm radial maze was located to one side of a 2.3 x 2.8 m room. The experimenter sat in one corner next to a table and an Apple MacIntosh SE computer. A "hypercard" stack was written for the computer to semi-automate the data recording and scoring. The room was illuminated by two 8 W fluorescent tube desk lamps positioned along the central axis of the maze on a shelf 2.15 m above the floor. These lamps provided a light intensity at the centre of the maze of 95 ± 5 lux. The background noise level in the room, mainly from the computer and the air conditioning, was measured at 57 - 63 dB.

2.1.3. Procedure

Hughes Exploration Box

At 42 to 48 days of age, each of the 51 experimental rats were tested once in one of the four exploration boxes. To control for possible differences in the boxes, near-equal numbers of rats from each condition and sex were tested in each of the four boxes. The testing took place over a four day period between 9 am and 1 pm. The testing order and side into which the rat was initially placed was balanced across conditions and sex to control for any possible day or time of day effects. To control for the possible effect of unfamiliar odour trails, male rats were always followed by female rats, and *vice versa*, and no two rats from the same cage were tested in the same exploration box. The day before the experiment began, eight rats which were not used in the

experiment were tested in the boxes to ensure unfamiliar odours were present for the first experimental rats. During the procedure, the experimenter was blind as to the experimental condition of the rats. All the rats which were required for each day of testing were transported in their home cages to the testing room before testing began.

Testing began with the two guillotine doors in place to divide the box into two separate halves. The rat was placed alone in one side of the box and the box lid and chamber door were closed. After 60 minutes, the rat was removed from the box to a holding container on top of the chamber, the guillotine doors were removed to allow access to the unfamiliar side of the box, and the rat was replaced in the quadrant of the box from which it was taken. The lid of the box and the door of the chamber were closed. Behavioural recording began about 30 seconds after the change-over operation was completed. Each rat was observed through the one way window a 10 minute period. During this period a record was made of:

(i) locomotor activity, based on a continuous cumulative record of the total number of quadrants entered by the rat. A quadrant was defined as entered if all four feet crossed into it.

(ii) whether the rat was (a) in the familiar or novel half of the box (novelty preference) and (b) whether it was rearing, grooming or engaging in neither of these behaviours. These measures were recorded at the end of five second intervals.

Radial Arm Maze

The rats were pretrained in the RAM during the weight reduction period described above. Pretraining continued until each rat had taken food at least once from every arm. Initially all doors were held open and a small quantity of the food reward was placed in every arm close to the centre of the maze. 'Nestles' chocolate 'Mini Morsels' were used as the reward. Gradually the food was available further down the arms to

the food wells and the raising and lowering of the doors was introduced. All rats were pretrained in eight sessions.

The rats were 64 - 67 days old when the first session of radial maze testing began. All 51 rats were tested for 40 sessions over a period of 68 days. The same testing order was used in all sessions. The order was balanced across groups for possible influences of time of day effects. The same relative pattern of baited and unbaited arms was used for all rats, but the pattern was rotated around the maze arms according to a randomly assigned rotation number, between 1 and 12, that was given to each rat. The first, third, fourth, seventh, eighth and nineth arms which followed the assigned rotation number arm, in a clockwise direction, were baited, while the other six arms were unbaited. This procedure was used to control for possible odour trails and differences in the complexity of remembering different bait patterns.

During the procedure, the experimenter was blind as to the experimental condition of the rats. For each session, a third of the rats were transported to the testing room at a time. Each trial began with the baiting of the correct arms for the rat, as displayed by the computer. The rat was placed in the centre of the maze for about five seconds before the doors were raised. When the rat entered an arm with four feet, all the doors were closed except for the one leading to the arm which the rat had entered. This door was closed when the rat returned to the centre of the maze. All doors remained closed for five seconds and then they were all re-opened.

Each time the rat entered an arm or the centre of the maze, the experimenter entered the rat's new location on the computer by pressing the appropriate key. The computer was programmed to record which type of response the rat had made and the time elapsed. Responses were coded as: initial entries to baited arms, re-entries to baited arms, initial entry to unbaited arms and re-entries to unbaited arms. Also, a computer generated 'beep' indicated the end of the 5 second 'doors-closed' period, and

the computer visually indicated when the rat had successfully completed the maze trial (eaten all rewards) or when the maximum time limit for the trial was over. The maximum time limit to obtain the six rewards was 15 minutes, after which the trial was terminated. If the trial was terminated and the rat had entered less than 15 arms, the data for that trial for that rat were excluded from analysis. If the rat had entered at least 15 arms, the data were included in the analysis, but only after it was adjusted to account for the number of baited arms that the rat had not visited. This adjustment involved (i) scoring the maximum of six for the initial entries to unbaited arms, and (ii) separately multiplying the number of re-entries to baited arms and re-entries to unbaited arms by the total number of baited arms (six) and dividing by the number of baited arms which were not entered.

Three types of error scores and two time measures were calculated for each rat on each trial. The error scores were (i) the number of initial entries to unbaited arms, (ii) the number of re-entries to unbaited arm, and (iii) the number of re-entries to baited arms. The time measures were (i) the mean time taken to visit each arm (total session time divided by the number of arms visited) and (ii) the mean latency to enter an arm after the doors were raised for the first six arm visits of the trial. Only six arm visits were considered as perfect performance on the maze required only six arm visits.

2.1.4. Statistical Analyses

Hughes Exploration Box

One sample t-tests were performed on the familiar and novelty preference measures for each group (Dose X Sex). Separate two-way analysis of variance (Dose X Sex) was performed on each of the novelty preference, rearing, grooming and ambulation measures.

Radial Arm Maze

For each rat, each of the three error scores and two time measures were averaged over blocks of five sessions. Each measure was then analysed by a three-way repeated measures analysis of variance (Dose X Sex X Blocks) with Blocks as the repeated measure. When significant treatment interaction was obtained, a one way analysis of variance (Dose) was performed on each Block with Neuman-Keuls tests of significance between paired means. Where litter effects were suspected, a similar one way ANOVA was performed with Neuman-Keuls test, with litter as the factor.

2.2. Results

Hughes Exploration Box

The effects of Dose on all behaviours is shown in Figure 2.1 (p. 46). Extreme cases were excluded from analysis, as defined by cases when the animal failed to enter the novel side of the exploration box, the rat apparently being asleep. For each experimental group (Dose X Sex), Table 2.2 (p. 47) shows the remaining group size and the mean and standard deviation of the ambulation score and observations of novelty preference, rearing and grooming. The one-sample t-tests for novelty preference (chance = 60) revealed a significant preference for novelty in the male control group, $t(7)=3.96$, $p<.01$, female control group, $t(7)=4.94$, $p<.01$, male 0.10% APM group, $t(8)=4.34$, $p<.01$, female 0.10% APM group, $t(7)=4.53$, $p<.01$ and the female 0.05% APM group, $t(8)=4.73$, $p<.01$. However the male 0.05% APM did not show a preference for novelty, $t(3)<1.0$, $p>.10$, and this may be a reflection of the small group size and that two of the rats in this group were excluded as extreme cases. The standard devaitions of the male 0.05% APM ($S.D.=33.2$) was considerably higher than the standard deviations of all other groups ($12.5 \leq S.D. \leq 20.7$) but the male 0.05% APM group did contain the two lowest scores out of all groups. A one-way ANOVA indicated that there was no litter effect on novelty preference, $F(7,38)<1.0$, $p>.10$.

Figure 2.1:
Hughes Exploration Box: Mean Five Second Observations of Novelty Preference, Rearing and Grooming, and Total Cells Entered for Control, 0.05% APM and 0.10% APM Groups.

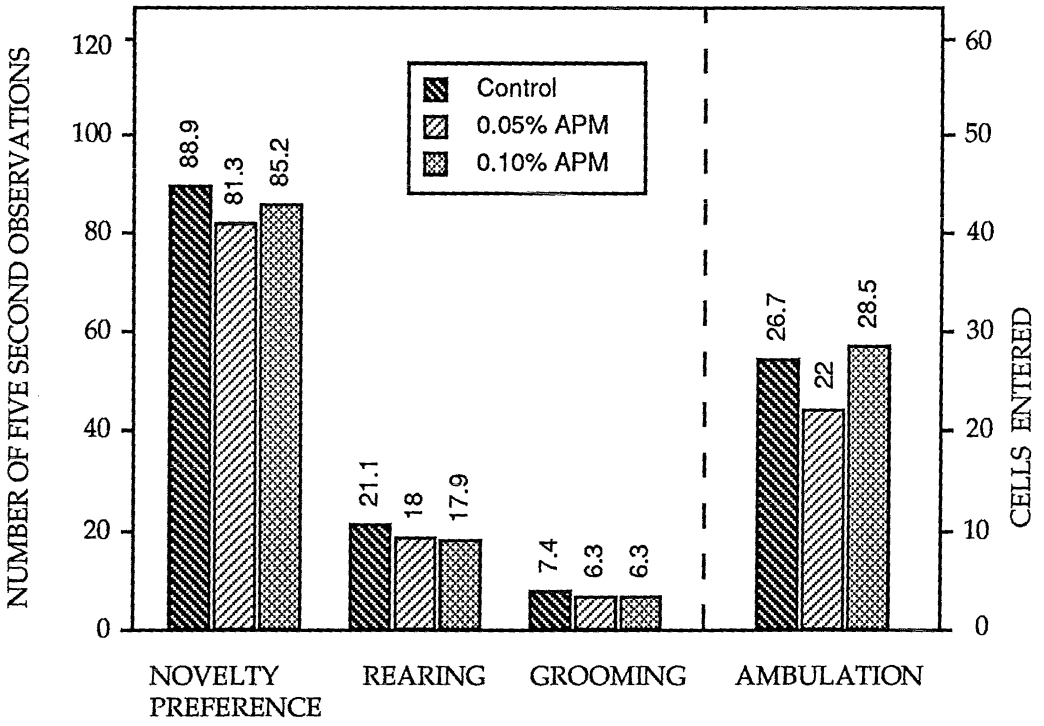


Table 2.3 (p. 47) shows the results of separate two way ANOVAs (Dose X Sex) for each dependent measure. These ANOVAs revealed no significant Dose effect on novelty preference, $F(2,40)=1.61$, $p>.10$, rearing $F(2,40)=1.7$, $p>.10$, grooming $F(2,40)<1.0$, $p>.10$ or ambulation $F(2,40)=1.52$, $p>.10$. Females showed a significantly greater preference for the novel side the of exploration box than males, $F(1,40)=8.35$, $p<.01$, but when the novelty preference of the control and 0.10% APM groups were compared with an unpaired t-test, the greater novelty preference displayed by the females was no longer significant, $t(31)=1.882$, $p=.069$. There were no other significant Sex effects, (F's<2.41, $p>.10$) and no significant Dose X Sex interaction effects (F's<1.06, $p>.10$).

Table 2.2
Remaining Group Sizes, Means ± Standard Deviations For All Exploration Box Dependent Measures (Extreme Values Omitted)

Measure	Group	Males	Females	Overall
n	Control	8	8	16
	0.05% APM	4	9	13
	0.10% APM	9	8	17
Novelty Pref.	Control	85.4 ± 18.1	92.5 ± 18.6	88.9 ± 18.1
	0.05% APM	61.3 ± 33.2	90.2 ± 19.0	81.3 ± 26.0
	0.10% APM	78.1 ± 12.5	93.4 ± 20.7	85.2 ± 18.0
Rearing	Control	20.6 ± 7.6	21.6 ±3 .0	21.1 ± 5.6
	0.05% APM	13.8 ± 4.7	19.9 ± 8.7	18.0 ± 7.9
	0.10% APM	16.9 ± 6.7	19.0 ± 5.4	17.9 ± 6.0
Grooming	Control	5.4 ± 3.0	9.4 ± 8.9	7.4 ± 6.7
	0.05% APM	7.8 ±6 .7	5.7 ± 5.0	6.3 ± 6.0
	0.10% APM	6.4 ± 8.2	6.1 ± 4.4	6.3 ± 6.5
Ambulation	Control	26.4 ± 9.9	27.0 ± 8.2	26.7 ± 8.8
	0.05% APM	22.0 ± 5.8	22.0 ± 9.0	22.0 ± 7.5
	0.10% APM	30.3 ± 11.4	26.5 ± 10.9	28.5 ± 11.0

Table 2.3
Summary of ANOVA Results For All Exploration Box Dependent Measures

Measure	Source	df:	*F*	*p*
Novel Pref.	Dose	2,40	1.61	0.2134
	Sex	1,40	8.35	0.0062 **
	Sex x Dose	2,40	1.06	0.3554
Rearing	Dose	2,40	1.70	0.1956
	Sex	1,40	2.41	0.1282
	Sex x Dose	2,40	0.55	0.5831
Grooming	Dose	2,40	0.12	0.8906
	Sex	1,40	0.07	0.7914
	Sex x Dose	2,40	0.82	0.4497
Ambulation	Dose	2,40	1.52	0.2304
	Sex	1,40	0.13	0.7192
	Sex x Dose	2,40	0.25	0.7787

Note: ** $p < .01$

Radial Arm Maze

Twenty-three cases (1.1% of all cases) were excluded from the analysis; 22 cases were excluded as the sessions were terminated and the rats had visited less than 15 arms of the maze and one case was excluded as a female 0.10% APM rat had a seizure in the maze. Table 2.4 shows the groups that the excluded cases came from. Note that each case refers only to one trial of one rat.

Table 2.4

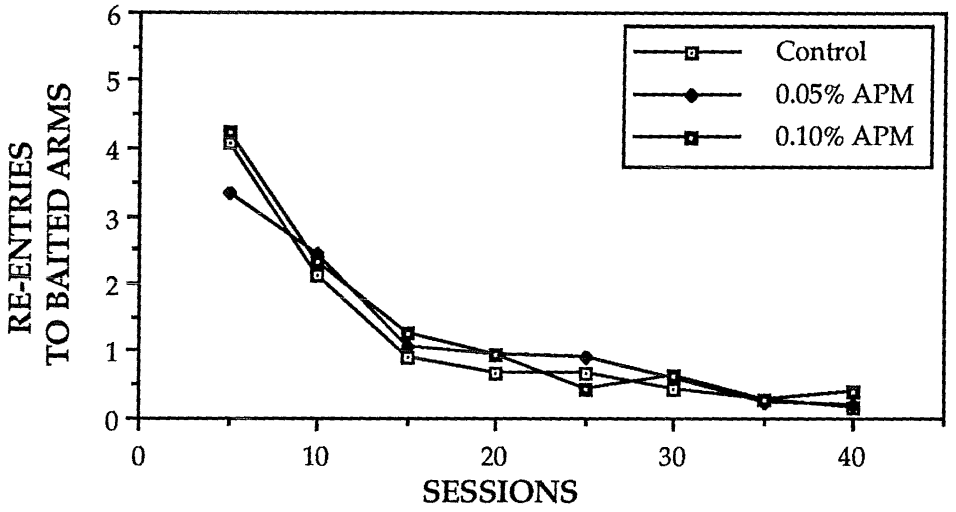
Radial Arm Maze Cases Excluded from the Analysis by Group

Group	Male	Female
Control	1	5
0.05% APM	4	10
0.10% APM	1	2

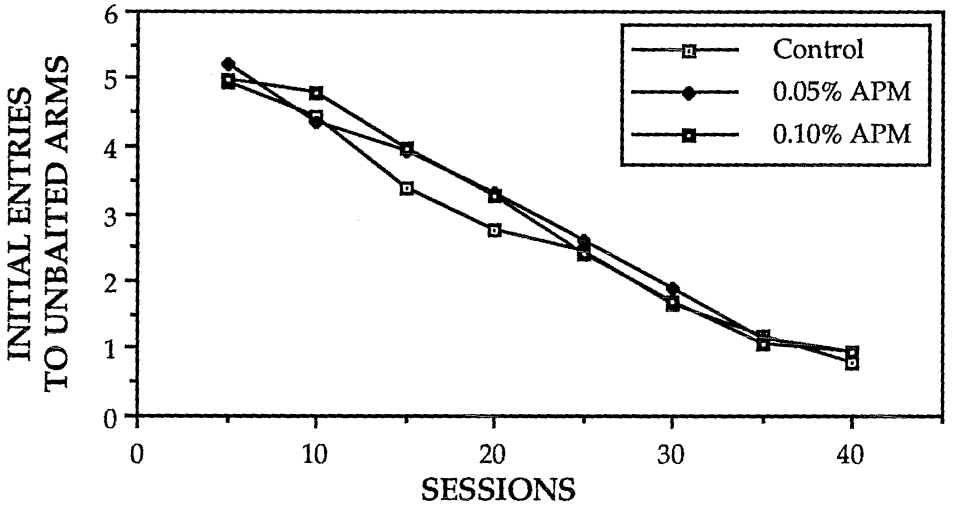
The effects of Dose and Sex on the three error scores are shown in Figures 2.2 (p. 49) and 2.3 (p. 50). Table 2.5 (p. 51) shows a summary of the three way ANOVAs (Dose X Sex X Blocks) with repeated measures (Blocks). The ANOVAs show that APM had no effect on the number of re-entries to baited arms, the number of initial entries to unbaited arms or the number of re-entries to unbaited arms. For these three error scores, there was no effect of Dose, $F(2,45)<1.00$, $p>.10$, Sex, $F(1,45)\leq 3.13$, $p<.10$, and no Dose X Sex interaction, $F(2,45)<1.00$, $p>.10$, although there was an indications that Sex tended to affect initial entries to unbaited arms, $F(2,45)=3.13$, $p<.084$. Within subjects, all three error scores significantly decreased across sessions, $F(7,315)\geq 128.87$, $p<.001$. However, there were no significant interaction effects of Dose X Blocks, $F(14,315)\leq 1.32$, $p>.10$, Sex X Blocks, $F(7,315)<1.00$, $p>.10$, or Dose X Sex X Blocks, $F(14,315)\leq 1.00$, $p>.10$. These analyses and Figure 2.2 combine to shows that, by the end of the experiment, all groups were making few errors and had learned the maze task to a high degree of proficiency. Also, the rate of acquisition of the RAM task was not significantly influenced by Dose or Sex.

Figure 2.2.
Radial Maze Error Scores by Dose

(a)



(b)



(c)

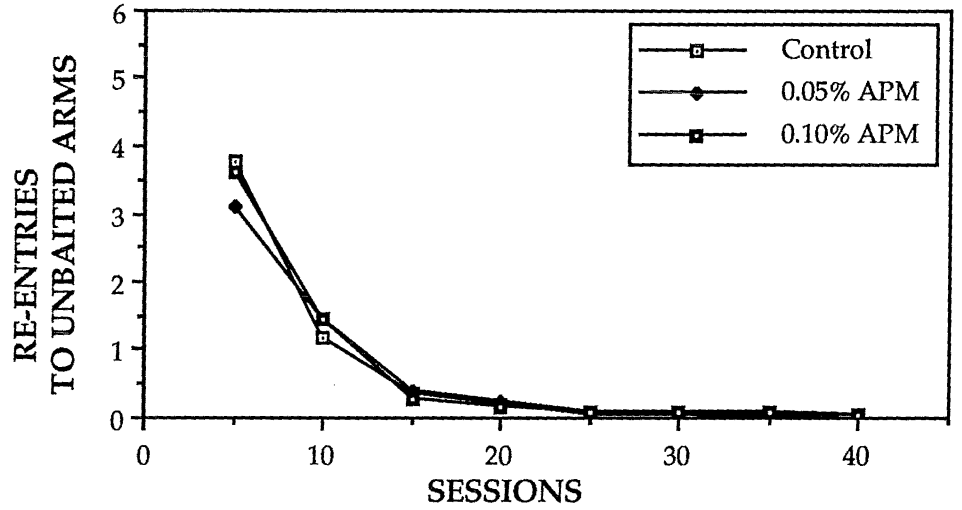
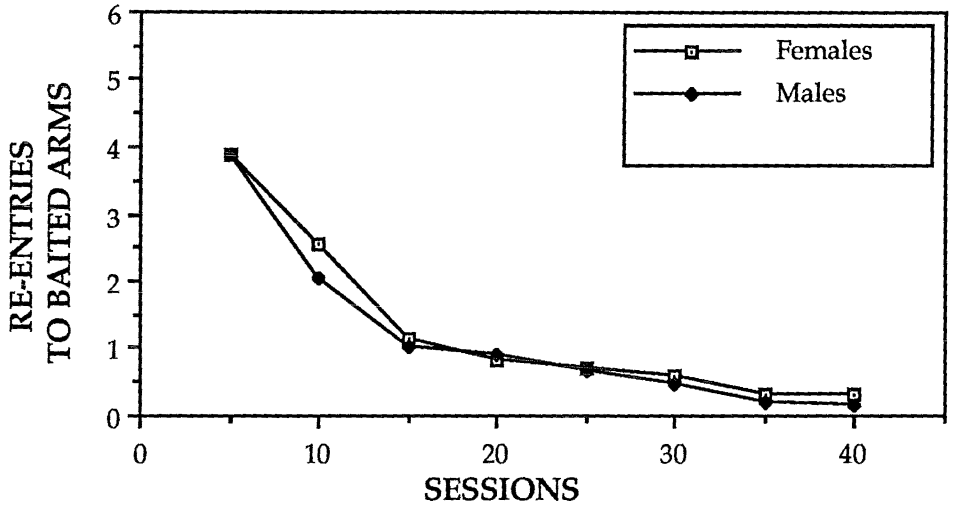
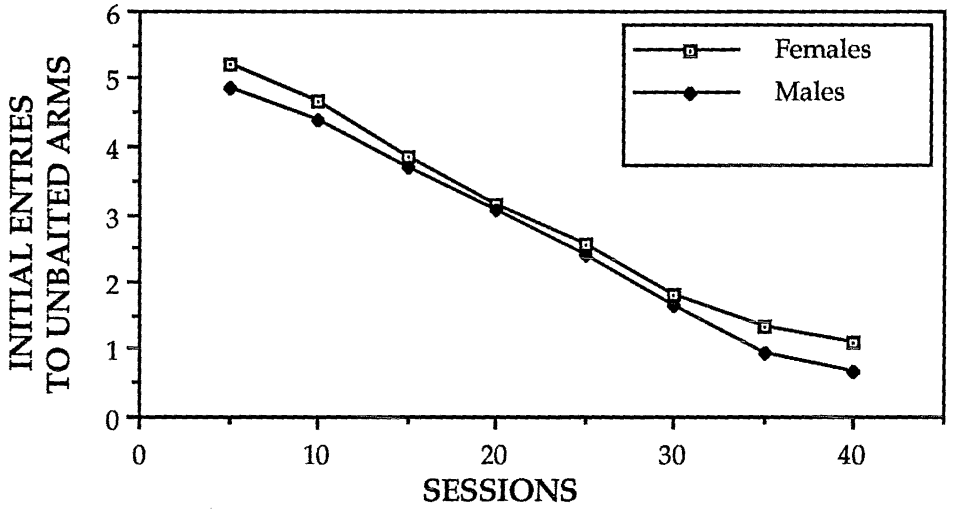


Figure 2.3.
Radial Maze Error Scores by Sex

(a)



(b)



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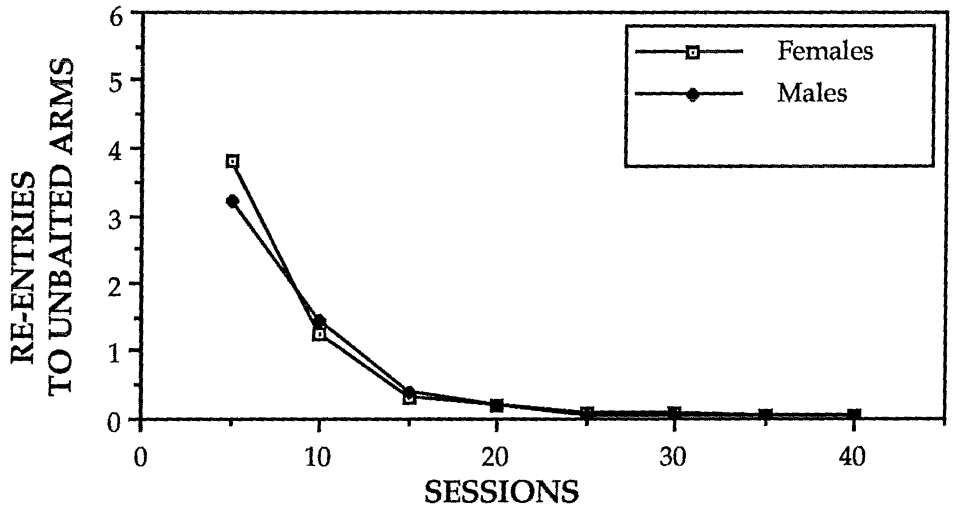


Table 2.5
Summary of ANOVA for Radial Arm Maze Error Scores

Measure	Source	df:	*F*	p
Re-entries	Between Subjects			
To Baited	Dose	2,45	0.62	.543
Arms	Sex	1,45	1.34	.253
	Dose X Sex	2,45	0.64	.534
	Within Subjects			
	Blocks	7,315	144.60	.000 **
	Dose X Blocks	14,315	1.32	.192
	Sex X Blocks	7,315	0.71	.665
	Dose X Sex X Blocks	14,315	0.59	.870
Initial Entries	Between Subjects			
To Unbaited	Dose	2,45	0.99	.380
Arms	Sex	1,45	3.13	.084
	Dose X Sex	2,45	0.48	.625
	Within Subjects			
	Blocks	7,315	251.70	.000 **
	Dose X Blocks	14,315	0.99	.463
	Sex X Blocks	7,315	0.40	.905
	Dose X Sex X Blocks	14,315	0.91	.543
Re-entries	Between Subjects			
To Unbaited	Dose	2,45	0.19	.831
Arms	Sex	1,45	0.23	.633
	Dose X Sex	2,45	0.72	.490
	Within Subjects			
	Blocks	7,315	128.87	.000 **
	Dose X Blocks	14,315	0.71	.763
	Sex X Blocks	7,315	0.78	.602
	Dose X Sex X Blocks	14,315	1.00	.458

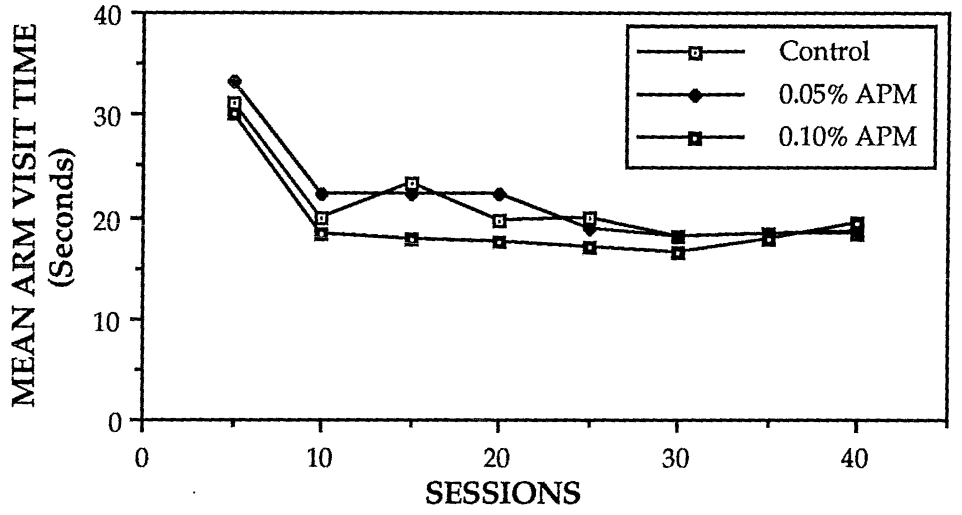
Notes: ** $p<.01$

Figure 2.4 (p. 53) shows the mean arm visit time expressed as a function of Dose and Sex. Table 2.6 (p. 54) shows a summary of the three way ANOVAs (Dose X Sex X Blocks) with repeated measures (Blocks). For the mean arm visit time measure, there was a signifcant between-subjects effect of Dose, $F(2,45)=3.40$, $p<.05$, and a significant within-subjects Dose X Blocks interaction, $F(14,315)=2.71$, $p<.01$. Neuman-Keuls tests of significance between rank ordered means ($\alpha=.05$) were performed on the mean of the arm visit times over all 40 sessions and revealed that the 0.10% APM group took significantly less time in the arms that the 0.05% APM group, but the mean time taken

by the control group was between the 0.10% APM and 0.05% APM groups and was non-significantly different from either. A one way ANOVA performed on the average time scores over the each of the blocks of sessions revealed a significant effect of Dose on the second, third and fourth blocks of sessions (respectively sessions 6-10, 11-15, 16-20), $F(2,48)\geq 3.90$, $p<.05$. Neuman-Keuls tests of significance between rank ordered means ($\alpha=.05$) showed that the 0.10% APM group took significantly less time to visit the arms than the 0.05% APM group on blocks two, three and four and the the 0.10% APM group took significantly less time than the control group on block three. Also, the control group took significantly less time than the 0.05% APM group on block two. There were no other significant effects of this measure. Taken together, there results suggest the time taken to visit the arms decreased to a fairly stable period by the end of the tenth session, and that the significant Dose X Blocks interaction was largely due to the 0.05% APM group taking longer than the 0.10% APM group on some of the early blocks of sessions. A one-way ANOVA of the mean arm visit times over all 40 sessions by litter revealed that the litter failed to reach an acceptable level of significance, $F(7,43)=2.17$, $p=.056$ and, more importantly, Neuman-Keuls tests showed that while one 0.05% APM litter took significantly longer than one control group litter and one 0.10% APM group litter, none of the litters were significantly different from any of the other litters in the same Dose. This suggests that the observed effects can not be attributed solely to genetic differences between the groups

Figure 2.4.
Mean Arm Visit Time in Radial Arm Maze by Dose and Sex

(a)



(b)

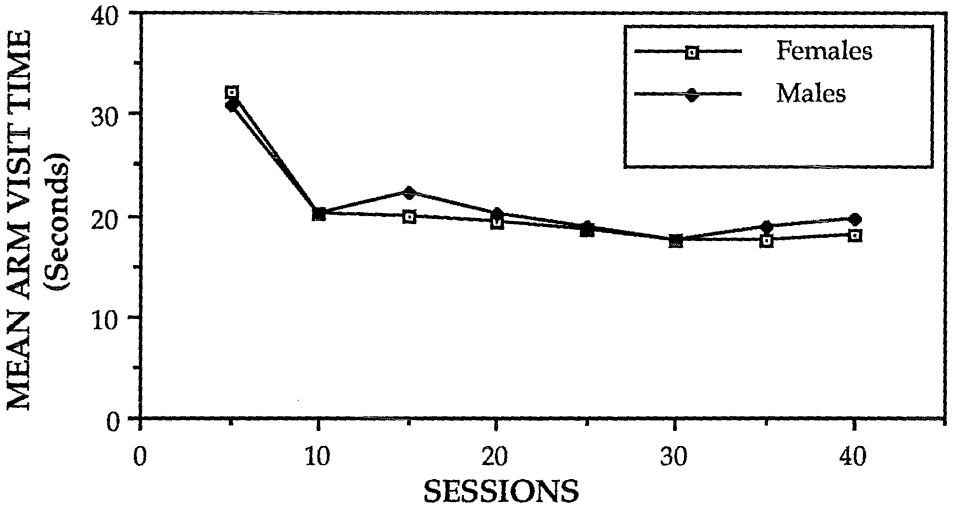


Table 2.6
Summary of ANOVA for Radial Arm Maze Time Measures

Measure	Source	df:	*F*	p
Mean Visit Time	Between Subjects			
	Dose	2,45	3.40	.042 *
	Sex	1,45	0.65	.426
	Dose X Sex	2,45	2.76	.074
	Within Subjects			
	Blocks	7,315	102.62	.000 **
	Dose X Blocks	14,315	2.71	.001 **
	Sex X Blocks	7,315	1.60	.136
	Dose X Sex X Blocks	14,315	0.86	.607
Arm Choice Latency (first six entries)	Between Subjects			
	Dose	2,45	4.80	.013 *
	Sex	1,45	0.00	.997
	Dose X Sex	2,45	0.22	.805
	Within Subjects			
	Blocks	7,315	33.76	.000 **
	Dose X Blocks	14,315	2.67	.001 **
	Sex X Blocks	7,315	1.03	.411
	Dose X Sex X Blocks	14,315	0.85	.610

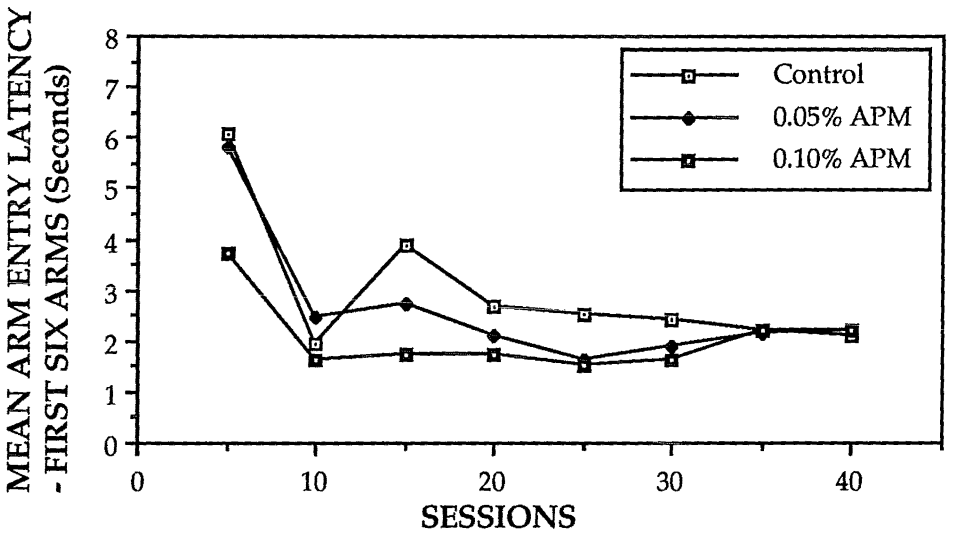
Notes: * $p<.05$
** $p<.01$

Figure 2.5 (p. 56) shows the group means for the mean arm entry latency, after the doors were opened for the first six arm entries, by Dose and Sex. Table 2.6 shows a summary of the three way ANOVAs (Dose X Sex X Blocks) with repeated measures (Blocks). On the arm entry latency measure, there was a signifcant between subjects effect of Dose, $F(2,45)=4.80$, $p<.02$, and a significant within subjects interaction effect of Dose X Blocks, $F(14,315)=2.67$, $p<.01$. Neuman-Keuls tests of significance between rank ordered means ($\alpha=.05$) were performed on the mean of the arm entry latencies over all 40 sessions and revealed that the 0.10% APM group took significantly less time to enter an arm that the control group, but the mean time taken by the 0.05% APM group was between the control and 0.10% APM group times and was non-significantly different from either. A one way ANOVA performed on these latency scores over each of the blocks of sessions revealed a significant effect of Dose on the first four blocks of

sessions, $F(2,48) \geq 3.33$, $p < .05$. Neuman-Keuls tests of significance between rank ordered means ($\alpha = .05$) showed that the 0.10% APM took significantly less time to enter the first six arms than the control group on the first, third and fourth blocks and the 0.10% APM took significantly less time than the 0.05% APM group on the first, second and third blocks. The 0.05% APM took significantly less time than the control group on the third block. Although Figure 2.5 (a) shows the the controls took longer to enter the arms on blocks five and six than the APM groups, neither the ANOVAs nor the Neuman-Keuls tests showed that these differences were significant. There were no other significant effects of this measure. Taken together, there results suggest that the mean latency to enter the first six arms decreased quickly for all groups but then the time taken by the control group increased slightly before becoming non-significantly longer than the APM groups for most of the remaining sessions. The significant Dose X Blocks interaction was largely due to the 0.10% APM group taking less time to enter the arms than the control groups over the first six blocks.

Figure 2.5.
Mean Arm Entry Latency for First Six Arm Entries in Radial Arm Maze by Dose and Sex

(a)



(b)

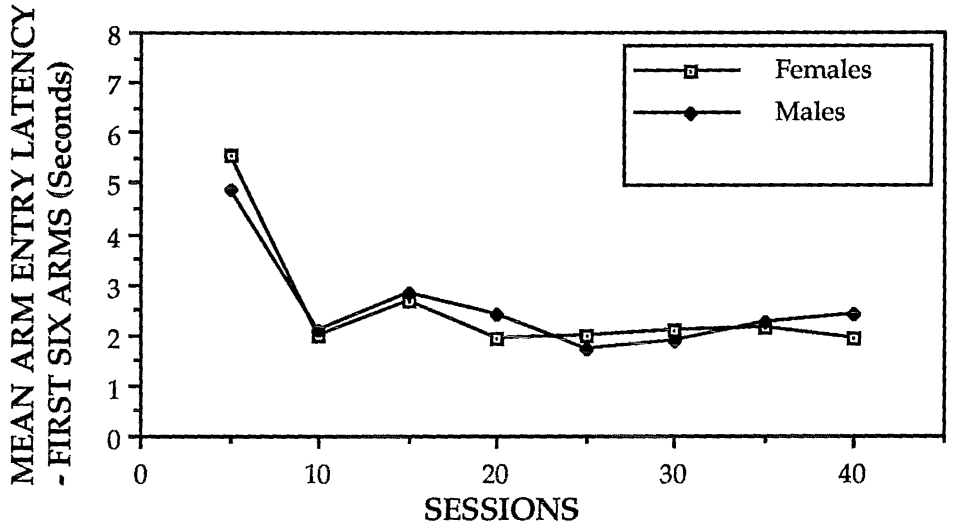


Figure 2.6 (p. 58) shows the weights of the rats by Dose and Sex and Table 2.7 shows a summary of the three way ANOVAs (Dose X Sex X Blocks) with repeated measures (Blocks). Figure 2.6 (a) suggests that the 0.05% APM group weights may have been slightly lower than the weights of the control and 0.10% APM groups, but this is probably a reflection of there being relatively fewer of the heavier males in the pooled male and female 0.05% APM group compared to the control and 0.10% APM groups. A three way ANOVA (Dose X Sex X Blocks) with Blocks as a repeated measure showed that the weights of the rats were not affected by Dose, either between subjects (Dose X Weight, $F(2,45)=1.861$, $p>.10$) or within subjects (Dose X Blocks, $F(14,315)<1.0$, $p>.10$, Dose X Sex X Blocks, $F(14,315)<1.0$, $p>.10$). There was a significant between subjects effect of Sex on the weight of the rats, $F(2,45)=261.36$, $p<.001$, and a significant within subjects interaction effect of Sex X Blocks, $F(14,315)=24.35$, $p<.001$. These results show that the male rats gained significantly more weight than the females, but the weight gain of either sex was not affected by the Dose condition of the rats.

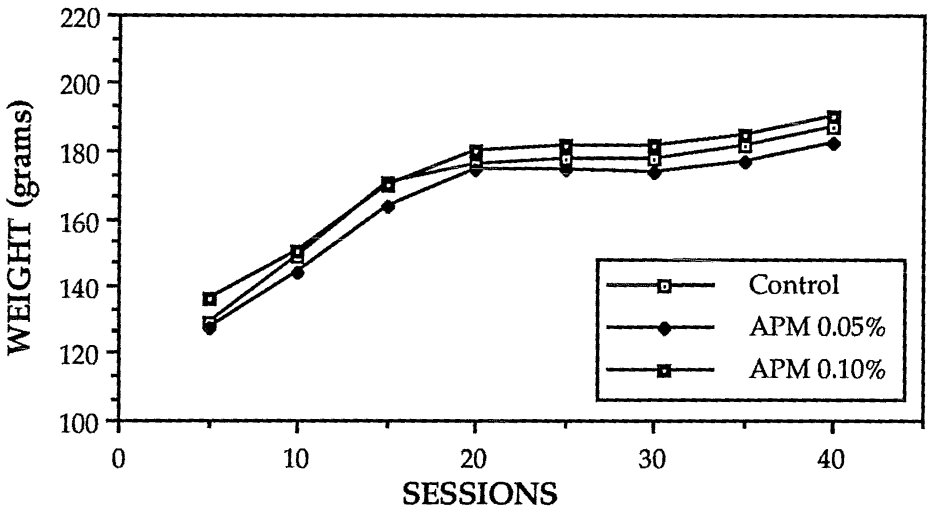
Table 2.7
Summary of ANOVA for Body Weights During Radial Arm Maze Testing Period

Measure	Source	df:	*F*	p
Body Weight	Between Subjects			
	Dose	2,45	1.861	.167
	Sex	1,45	261.34	.000 **
	Dose X Sex	2,45	2.373	.105
	Within Subjects			
	Blocks	7,315	642.81	.000 **
	Dose X Blocks	14,315	0.70	.773
	Sex X Blocks	7,315	24.35	.000 **
	Dose X Sex X Blocks	14,315	0.26	.997

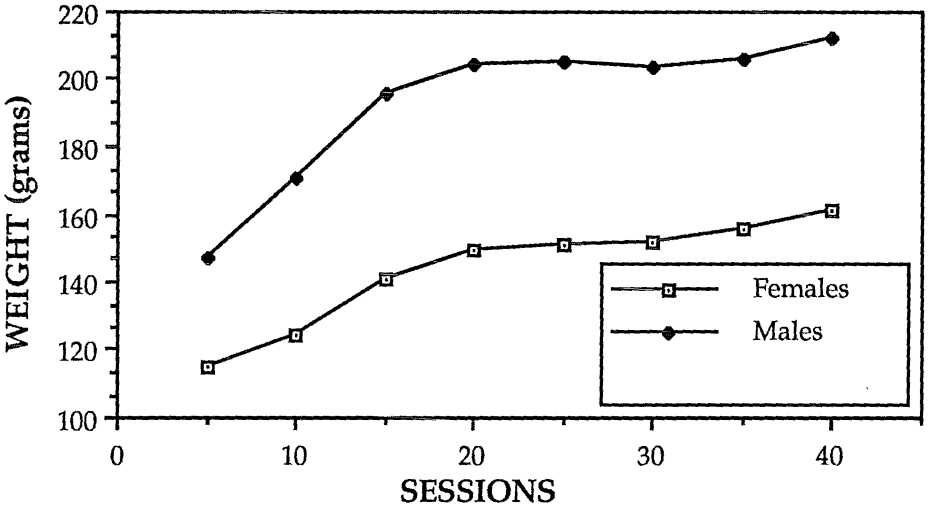
Notes: ** $p<.01$

Figure 2.6.
Weight During Radial Arm Maze Testing Period by Dose and Sex

(a)



(b)



Chapter 3

Discussion

3.1. Main Findings

3.1.1. Hughes Exploration Box

The results of the present study show that prenatal exposure to low doses of APM (60 and 120 mg/Kg/day) does not affect exploratory behaviour in rats, as measured in the Hughes exploration box. The male 0.05% APM group was the only group not to show a significant novelty preference, and the larger standard deviations of this group, compared to all other groups, suggests that this may be partly due to the very small remaining group size (n=4) after two of the rats in this group were excluded as extreme cases. However, this group did contain the lowest novelty scores across any group and so the increased variance does not adequately explain the finding of a lack of novelty preference in the male 0.05% APM group. When considering both APM groups and the control group, females displayed a significantly greater novelty preference than males, but when considering only the control and 0.10% APM groups, this trend was no longer significant. This shows that the statistical difference between sexes was influenced by the low novelty preference of the 0.05% APM group. An analysis of this measure by litter showed that this effect was not due to a litter effect and it remains unclear why this male group scored so lowly on the novelty measure. There is no reason to discount the sex effect, especially as the trend towards the effect existed when the 0.05% APM groups were not considered, even though the trend did not quite reach statistical significance.

As females displayed both a significantly greater novelty preference and unchanged ambulation scores, it can be can be concluded that they displayed more exploratory behaviour than males. This is an unusual finding and, to the knowledge of the author, there are no reports of similar findings in the literature[3]. The results of this study are also unusual in that they do not support the typical observations in the Hughes exploration box that females are more active and rear more than males (e.g., Hughes and Pither, 1987; Hughes and Swanberg, 1970) and males groom more than females (Hughes, 1970). However a sex difference in grooming is not consistently reported in the literature (e.g., Hughes and Pither, 1987) and other differences between the present study and the literature may possibly be related to the age of the animals in the present study (42-48 days). But, while Hughes (1970) reported no sex difference between grooming in young rats (M=47 days), he also reported no sex difference on novelty preference and that females had higher ambulation scores than males, although ambulation scores were lower for young animals than older animals (M=189 days). Clearly, age effects do not account for all of the unusual findings in the exploration box.

3.1.2. Radial Arm Maze - Error Scores

The present study does not support the hypothesis that the prenatal exposure of rats to the low doses of APM used in this experiment may impair spatial memory in the RAM. On the RAM test, there were no dose, sex or interaction effects on any of the three error scores: re-entries to baited arms, initial entries to unbaited arms and re-entries to unbaited arms. By the last block of sessions, all groups were making few of any of the classes of errors and hence all groups showed that they had learned the task to a high degree of proficiency. While the shape of the learning curves depicted by the error scores across blocks of sessions were not subjected to statistical analysis,

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[3] Hughes, personal communication.

the initial entries by unbaited arms errors appear to decrease in a linear fashion whereas the re-entries to both baited and unbaited arms appear to decrease at a more exponential or hyperbolic rate. These somewhat tenuous observations could be interpreted as supporting the notion that RAM performance is dependent on two different types of memory and that the same type of memory is required for making re-entries to both baited and unbaited arm while a different type of memory is required for making initial entries to unbaited arms. This would support the working and reference memory distinctions made by Jarrard (1986).

3.1.3. Radial Arm Maze -Time Effects

Arm Visit Time

The results revealed that prenatal exposure to low doses of APM affected the time taken by rats to complete some aspects of the RAM task. For the mean arm visit times, the results of the one way ANOVAs and the Neuman-Keuls tests of significance on the means of all 40 sessions and the eight blocks of sessions suggest that the interaction of dose by blocks was largely due to the 0.05% APM group taking longer than the 0.10% APM group on some of the early blocks of sessions. While Figure 2.4 (a) (p. 53) shows the 0.10% APM took less time in the arms than the other groups, the means of the control and 0.10% APM groups differ significantly on only one block of sessions and not across the mean score of all 40 sessions. This discounts the possibility of a simple dose-response effect on this measure. One explanation for this observed difference between the 0.05% APM group and other groups is that the reduced genetic variability in the 0.05% APM groups could exert itself as a difference between litters on this measure. The analysis of the mean arm visit times of the 40 sessions by litter revealed an non-significant trend towards litter differences and the Neuman-Keuls tests revealed that the none of the litter means were significantly different from any other litters of the

same Dose. However, the small number of litters in each group, and especially the 0.05% APM group, would make it very hard to separate a litter effect from a dose effect. Nevertheless, litter differences do not seem to explain the unusual relationship between dose and mean arm entry time.

Arm Entry Latency

The results for the initial arm entry latencies appear to exhibit a simple dose-response relationship but are not easily explained in terms of behavioural mechanisms. The one way ANOVAs and Neuman-Keuls tests for the blocks of sessions suggest that the interaction effect of dose by blocks was largely due to the 0.10% APM groups taking significantly less time to enter the arms than the control groups over the first half of the blocks. A similar effect over the later half of the experiment may have been obscured as the time taken by all rats aproached an asymptote and the relative significance of the error created by the reaction time of the experimenter increased. During five of the first six blocks, the arm entry latency for the 0.05% APM groups fell between the control and 0.10% APM groups and on two of these occasions the 0.05% APM latency was significantly different from either one or both of the control and 0.10% APM groups. Taken together, these findings suggest a dose-response relationship on the arm entry latency measure.

A behavioural interpretation of the arm entry latency finding is difficult and, to the knowledge of this author, no previous studies have recorded this aspect of RAM performance. One possible explanation of this finding would be that the 0.10% APM rats were more motivated than the control rats to search for food in the maze, but the results show that weight did not vary across dose. It is possible that the rats exposed to APM were hungrier despite their equal body weights but the Brunner *et al.* (1979) study reported no difference in the body weights of the free feeding offspring of rats whose mothers had exposed to up to 2 g/Kg/day APM throughout gestation. If APM

did have a motivational effect on the rats, this in itself would be an interesting treatment effect.

It is noteworthy that over the first four blocks of sessions, the 0.10% APM group showed shorter arm entry latencies than the control group but over the same blocks the mean arm visit time of the 0.10% APM group did not appear to be consistently longer than that of the control group. This, together with the lack of dose effect on ambulation in the exploration box, suggests that the 0.10% APM group were not just exhibiting an increased level of locomotor activity. Instead, the difference may reflect a difference in emotional reactivity to the sudden change in reward contingencies from the pretraining to the testing phases of the RAM task. During pretraining, several pieces of food were available at each arm but when the testing sessions began only one piece of food was available at only half of the arms. A rat which was more reactive to this sudden change in contingencies may have been adapted more quickly to the novel task of choosing between arms. However the lack of difference in the average time taken to visit the arms once again suggests that this is an inadequate explanation of the results.

The results show that the 0.10% APM group took less time to enter the first six arms of each trial than the control group yet the analysis of the error scores in the RAM shows that the 0.10% APM group made no more errors than the control group. As the 0.10% APM group learned the RAM task as well as control group but took less time to choose the correct arms, it is tempting to speculate that this reflects an enhancement of the maze learning abilities of the 0.10% APM group. Indeed, ASP, and its structural and functional analogue GLU, are involved in the neurobiology of memory and recent research has suggested that low doses of these excitatory amino acids may enhance memory development and function. Low doses of MSG have been found to enhance memory function in some individuals with Alzheimers' disease (see Deutsch and Morihisa, 1988) and low dose MSG has been found to promote neuronal growth and

differentiation, *in vitro*, in neural cell cultures (Aruffo, Ferszt, Hildebrandt and Cervos-Navarro, 1987). Hence it is possible that prenatal exposure to low doses of excitatory amino acids, such as ASP and GLU, may *enhance* the development of neural systems involved in memory. However, within the context of prenatal exposure to APM, the animal is also exposed to PHE which is likely to exert a dose-dependent impairment of cognitive functions. Perhaps the effects of low doses of ASP and PHE may counter act each other to obscure any overall effect. If so, this could conceivably account for the lack of observed effect on RAM performance in the present study. This hypothesis could be investigated by future research which separately examines the effects of prenatal exposure to PHE and sub-excitotoxic doses of ASP or GLU on RAM performance.

3.2. Contribution of the Present Study

The results of the present study suggest that prenatal exposure to a low dose of APM reduces the arm entry latency of the first six arm visits in the 12-arm radial arm maze in rats. To the knowledge of the current author, this tentative finding is the first report of a behavioural effect of prenatal exposure to a low dose of APM. The only previous behavioural teratology studies of APM which the present author found (Mahalik and Gautieri, 1984; Brunner *et al.* , 1979) gave high doses of APM to the rat dams (between 1 and 5 g/Kg/day) and found developmental delays of the offspring. However, both of these studies failed to use adequate cross-fostering procedures and therefore their findings could be due to an indirect effect of APM on the dams. Indeed, Brunner *et al.* (1979) cross-fostered control offspring to APM fed dams and the postnatal developmental delays in these animals led them to attribute the findings of their teratology study to the effects of postnatal exposure to APM. The present study is also the first behavioural teratology study of APM which employed an adequate cross-fostering procedure or a low dose of APM.

It has previously been believed that low doses of APM are unable to affect the fetus because of the natural occurrence of APM's metabolites (ASP, PHE and methanol), the inability of ASP to cross the mature placenta and the presumed inability of the intact peptide even to enter the mother. However, the tentative finding in the present study of a dose-response effect on arm entry latencies suggests that APM and/or its metabolites do reach the fetus in sufficient quantities to induce some kind of functional change. It is well beyond the scope of the present study to decipher whether the offending agent is PHE, ASP or the intact APM peptide or to confirm or refute the hypothesis that APM released ASP may be able to pass through the undeveloped placental barrier.

To convert the dose of APM consumed by the rat dams in this study to a human equivalent, the rat dose must be divided by six to account for the faster metabolic rate of rats (Pardridge, 1986). Therefore, the human dose equivalent to the 0.10% APM groups' consumption is about 120 mg/Kg/day ÷ 6 = 20 mg/Kg/day : less than two thirds of the American FDA expected daily intake figure! It must be cautioned that the findings of the present study may be due to chance. Clearly, the present study is urgently in need of replication. If the results of the present study are found to be a consistent effect of prenatal exposure to low doses of APM, then APM may be exerting a widespread but unrecognized effect of public health.

3.3. Limitations of the Present Study

The present study employed a range of control procedures which were found lacking in previous behavioural teratology studies in the literature. The present study employed adequate cross-fostering procedures to control for indirect maternal effects, the experimenter was blind to experimental condition of the rats, and testing orders on

both behavioural tests were alternated across Dose and Sex to balance for any possible circadian variations in behaviour. Both of the behavioural tests employed in the present study are well validated measures. In the Hughes exploration box, the familiar side of the box and the particular box used were balanced across dose and sex. In the RAM, response habits were controlled by using doors and possible extra-maze or intra-maze stimulus differences were balanced by randomly rotating the pattern of baited and unbaited arms around the maze. The same bait pattern was used in case different patterns were harder to learn than others. None the less, the findings of the present study must be considered within the context of its methodological problems and limitations. As noted in the method section, behavioural teratology studies should ideally take only one rat from each experimental and control group litter so that the genetic variability of the groups may be maximized and the problem of confounding genetic and prenatal determinants of behavioural variation may be minimized. In an attempt to make the most of limited resources, the present study attempted to take a maximum of three males and three females from three different litters to make experimental groups of nine rats with a reasonable degree of genetic diversity. However, the failure of two of the four 0.05% APM dams to produce viable litters and the birth of only female pups to another 0.05% APM dam resulted in the 0.05% APM groups coming from only two litters, one of these litters contributing 11 of the total of 51 experimental rats and the male 0.05% APM group having only six rats. Hence the present study was a long way from the ideal one subject per litter. To some extent, these problems are lessened by the desired adequacy of the control and 0.10% APM groups, but it must be remembered that each of these groups is comprised of only three litters. It would be advisable for future studies to decrease the number of experimental subjects taken from each litter or increase the size of the experimental groups.

Behavioural teratogenic effects may interact with developmental changes in the animal to produce behavioural impairment at one age of the animal but not at a different age. Thus, the findings of the present study are weakened by testing the

animals at only one age. Ideally, behavioural teratology studies should take a more longitudinal approach to behavioural sampling and investigate the behaviour of different groups of animals at different stages throughout their life-cycle. Future research may examine whether the dose-response relationship observed in the present study between APM and arm entry latencies in the RAM is displayed by older animals.

Dose-response relationships in behavioural teratology are quite sensitive and relatively small changes in the dose of a teratogenic agent can change the nature of the observed response from no effect to a behavioural change, to a morphological change or even to death (Vorhees, 1986). As the dose zone for any particular effect is narrow, a further short-coming of the present study is that it used only two low dose levels and hence it was unlikely to reveal the dose-zone for any particular behavioural effect. However the tentative finding of a dose response relationship between APM and arm entry latencies suggests that the higher dose in the present study may represent the lower end of the dose zone for this effect. As the literature on phenylketonuria and excitotoxic amino acids show, respectively, that high doses of PHE and most probably ASP can exert teratogenic effects on rats in a range of experimental learning tasks (see Sections 1.3.2, 1.5), it is most likely that the lack of observed effect on spatial memory was due to the doses of APM used in the present study being too low to impair performance on the RAM task.

The RAM and the Hughes exploration box are well validated robust tests that have been used previously in behavioural teratology studies (Hughes and Beveridge, 1987; Adams, 1986). From these tests, it can be safely assumed that prenatal exposure to the low doses of APM used in the present study produces no impairment in spatial memory or changes in exploratory behaviour in young rats. However, these negative findings must also be considered within the context of the methodological limitations of the present study and these negative findings do not allow the conclusion that exposure to low doses of APM does not exert any other unmeasured behavioural

teratogenic effect. The inferences that can be made from a behavioural teratology study are limited to the empirically derived knowledge of what behaviours the tests have measured, the degree of damage required to the relevant neural system before these behaviours are affected and the extent that the animal may be able to compensate for any specific deficit. The possibility always remains that a behavioural effect has gone unobserved. While Vorhees and Butcher (1982) correctly point out that an exhaustive invesigation of the behavioural teratogenicity of a substance could virtually last forever, the striking lack of methodologically sound behavioural teratology studies with APM warrant considerable future research.

3.4. Recommendations for Future Studies

The experience gained in the present study suggests that future behavioural teratology studies will benefit from increasing the genetic variability of the groups by taking fewer than six subjects from each litter. The tentative findings of this study support the value of low dose behavioural teratology studies, but future studies would be able to contribute more to their area of research by employing more dose levels which are more widely spaced from each other than those in the present study. This would more readily establish the dose zone for any particular response.

In the present study, the ability to measure the time spent in various parts of the maze was responsible for detecting the arm entry latency effect. However, time measurement was influenced by the response time of the experimenter. Also, the software which determined and recorded the time was only accurate to within one second. As the time taken by the rats to move around the maze decreased, these sources of uncontrolled, random error became more significant, and they may have helped to obscure a dose effect in the later blocks of the session. Future studies may overcome these problems by employing some kind of automated time and place measurement

system which is accurate to fractions of a second and does not rely on the response time of the experimenter. Perhaps a computer controlled automated RAM procedure, similar to that described most recently by Peele and Baron (1988), could be adapted to record separately the time elapsed in both the arms and the hub of the maze.

The necessary replication of the present study would benefit from employing additional measures which would allow a more thorough assessment of the behavioural teratogenic effects of APM. A range of measures may provide some understanding of the exact behavioural dimensions that have been observed in this study as a variation in the arm entry latency measure. For instance, if open field testing was employed before other tests in the battery, the naive rat's behaviour during the initial trial could provide a measure of emotional reactivity while the rats behaviour over a number of trials could provide a measure of general activity level.

As well as replication of the present study, the understanding of the behavioural teratogenic potential of APM would greatly benefit from more research in the following areas:

(i) Behavioural teratology studies of PHE using a demanding RAM procedure would help determine the lower limits of the dose zone of PHE's adverse effect on cognitive development. This would contribute to the understanding of the risks associated with hyperphenylalanemia as well as enabling a more accurate assessment of the likely effects of maternal APM consumption of the developing fetus.

(ii) Examining the effects of prenatal and postnatal exposure to ASP using a demanding RAM procedure would investigate the hypothesis that low doses of ASP or GLU may in fact facilitate the development of the neural systems involved in memory. Given the known adverse consequences of higher doses of excitatory amino acids, a thorough investigation would be required to establish the dose zones of the hypothesised beneficial effects and known adverse effects. Also, longitudinal designs would be recommended to determine whether the positive or negative effects of these

agents may vary over the age of the animal. If low doses of ASP can exert a beneficial effect of the developing neural systems of memory, then the effect of APM released ASP may mask the possible adverse effects of prenatal exposure to APM released PHE.

(iii) The biological investigation of the transfer characteristics of the undeveloped placenta would be of considerable benefit to most teratology investigations. Knowledge of the undeveloped placenta's transfer of ASP would enable more informed and hopefully more accurate predictions from the biological literature about the likely behavioural consequences of prenatal exposure to APM.

3.5. Concluding Comments

Despite the concern over the safety of APM, some of APM's protagonists believe that the number of complaints made about APM may stem from its high public profile as a synthetic sweetener (e.g., Moser, 1986, Kellen, 1987). They emphasize the benefits of APM to diabetic and overweight people and to all people who wish to avoid tooth decay. They note that food allergies are common to a range of foods and it may be unusual that so few problems are experienced with a food that is so widely consumed. However, only a handful of published studies have investigated the behavioural effects of APM, even though it is claimed to be the most thoroughly tested of all food additives, is currently used by over 100 million people and has been the target of over 4000 consumer complaints. The lack of published behavioural literature is a cause for concern, and this lack of literature impeded the adequate interpretation of the tentative findings of the present study; that prenatal exposure to even low doses of APM may affect RAM arm entry latencies even though the number of errors in the RAM may not be affected. Whether this finding is replicated and/or of relevance to the safety of APM will only be established by further research of both the biological and behavioural effects of APM.

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